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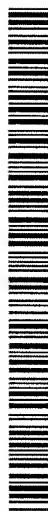
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(54) Title: DISEASE PREVENTION BY REACTIVATION OF THE THYMUS

(57) Abstract: The present disclosure provides methods for gene therapy utilizing hematopoietic stem cells, lymphoid progenitor cells, and/or myeloid progenitor cells. The cells are genetically modified to provide a gene that is expressed in these cells and their progeny after differentiation. In a preferred embodiment the cells contain a gene or gene fragment that confers to the cells resistance to HIV infection and/or replication. The cells are administered to a patient in conjunction with treatment to reactivate the patient's thymus. The cells may be autologous, syngeneic, allogeneic or xenogeneic, as tolerance to foreign cells is created in the patient during reactivation of the thymus. In a preferred embodiment the hematopoietic stem cells are CD34+. The patient's thymus is reactivated by disruption of sex steroid mediated signaling to the thymus. In a preferred embodiment, this disruption is created by administration of LHRH agonists, LHRH antagonists, anti-LHRH receptor antibodies, anti-LHRH vaccines or combinations thereof.

## DISEASE PREVENTION BY REACTIVATION OF THE THYMUS

## FIELD OF THE INVENTION

**[0001]** The present disclosure is in the field of disease prevention. In particular this invention is in the field of stimulating a patient's immune system through reactivation of the 5 thymus, and optionally using gene therapy of hematopoietic stem cells (HSC), hematopoietic progenitor cells, epithelial stem cells or bone marrow.

## BACKGROUND OF THE INVENTION

## THE IMMUNE SYSTEM

**[0002]** The major function of the immune system is to distinguish "foreign" antigens 10 from "self" and respond accordingly to protect the body against infection. In normal immune responses, the sequence of events involves dedicated antigen presenting cells (APC) capturing foreign antigen and processing it into small peptide fragments which are then presented in clefts of major histocompatibility complex (MHC) molecules on the APC surface. The MHC molecules can either be of class I expressed on all nucleated cells (recognized by cytotoxic T 15 cells (Tc)) or of class II expressed primarily by cells of the immune system (recognized by helper T cells (Th)). Th cells recognize the MHC II/peptide complexes on APC and respond; factors released by these cells then promote the activation of either of both Tc cells or the antibody producing B cells which are specific for the particular antigen. The importance of Th cells in virtually all immune responses is best illustrated in HIV/AIDS where their absence 20 through destruction by the virus causes severe immune deficiency eventually leading to death. Inappropriate development of Th (and to a lesser extent Tc) can lead to a variety of other diseases such as allergies, cancer and autoimmunity.

**[0003]** The ability to recognize antigen is encompassed in a plasma membrane 25 receptor in T and B lymphocytes. These receptors are generated randomly by a complex series of rearrangements of many possible genes, such that each individual T or B cell has a unique antigen receptor. This enormous potential diversity means that for any single antigen the body might encounter, multiple lymphocytes will be able to recognize it with varying degrees of binding strength (affinity) and respond to varying degrees. Since the antigen receptor specificity arises by chance, the problem thus arises as to why the body doesn't "self 30 destruct" through lymphocytes reacting against self antigens. Fortunately there are several mechanisms which prevent the T and B cells from doing so – collectively they create a situation where the immune system is tolerant to self.

[0004] The most efficient form of self tolerance is to physically remove (kill) any potentially reactive lymphocytes at the sites where they are produced (thymus for T cells, bone marrow for B cells). This is called central tolerance. An important, additional method of tolerance is through regulatory Th cells which inhibit autoreactive cells either directly or 5 more likely through cytokines. Given that virtually all immune responses require initiation and regulation by T helper cells, a major aim of any tolerance induction regime would be to target these cells. Similarly, since Tc's are very important effector cells, their production is a major aim of strategies for, e.g., anti-cancer and anti-viral therapy.

#### THE THYMUS

[0005] The thymus is arguably the major organ in the immune system because it is the primary site of production of T lymphocytes. Its role is to attract appropriate bone marrow-derived precursor cells from the blood, and induce their commitment to the T cell lineage including the gene rearrangements necessary for the production of the T cell receptor for antigen (TCR). Associated with this is a remarkable degree of cell division to expand the 10 number of T cells and hence increase the likelihood that every foreign antigen will be recognized and eliminated. A strange feature of T cell recognition of antigen, however, is that unlike B cells, the TCR only recognizes peptide fragments physically associated with MHC molecules; normally this is self MHC and this ability is selected for in the thymus. This 15 process is called positive selection and is an exclusive feature of cortical epithelial cells. If the TCR fails to bind to the self MHC/peptide complexes, the T cell dies by "neglect" – it 20 needs some degree of signaling through the TCR for its continued maturation.

[0006] While the thymus is fundamental for a functional immune system, releasing ~1% of its T cell content into the bloodstream per day, one of the apparent anomalies of mammals is that this organ undergoes severe atrophy as a result of sex steroid production. 25 This can begin even in young children but is profound from the time of puberty. For normal healthy individuals this loss of production and release of new T cells does not always have clinical consequences (although immune-based disorders increase in incidence and severity with age). When there is a major loss of T cells, as in AIDS and following chemotherapy or 30 radiotherapy, the patients are highly susceptible to disease because they are immune suppressed.

[0007] Many T cells will develop, however, which can recognize by chance, with high affinity, self MHC/peptide complexes. Such T cells are thus potentially self-reactive and

could cause severe autoimmune diseases such as multiple sclerosis, arthritis, diabetes, thyroiditis and systemic lupus erythematosis (SLE). Fortunately, if the affinity of the TCR to self MHC/peptide complexes is too high in the thymus, the developing thymocyte is induced to undergo a suicidal activation and dies by apoptosis, a process called negative selection.

5 This is called central tolerance. Such T cells die rather than respond because in the thymus they are still immature. The most potent inducers of this negative selection in the thymus are APC called dendritic cells (DC). Being APC they deliver the strongest signal to the T cells; in the thymus this causes deletion, in the peripheral lymphoid organs where the T cells are more mature, the DC cause activation.

10 **THYMUS ATROPHY**

[0008] The thymus is influenced to a great extent by its bidirectional communication with the neuroendocrine system (Kendall, 1988). Of particular importance is the interplay between the pituitary, adrenals and gonads on thymic function including both trophic (thyroid stimulating hormone or TSH, and growth hormone or GH) and atrophic effects (leutinizing hormone or LH, follicle stimulating hormone or FSH, and adrenocorticotropic hormone or ACTH) (Kendall, 1988; Homo-Delarche, 1991). Indeed one of the characteristic features of thymic physiology is the progressive decline in structure and function which is commensurate with the increase in circulating sex steroid production around puberty (Hirokawa and Makinodan, 1975; Tosi *et al.*, 1982 and Hirokawa, *et al.*, 1994). The precise target of the 15 hormones and the mechanism by which they induce thymus atrophy is yet to be determined. Since the thymus is the primary site for the production and maintenance of the peripheral T cell pool, this atrophy has been widely postulated as the primary cause of an increased incidence of immune-based disorders in the elderly. In particular, deficiencies of the immune system illustrated by a decrease in T-cell dependent immune functions such as cytolytic T-cell 20 activity and mitogenic responses, are reflected by an increased incidence of immunodeficiency, autoimmunity and tumor load in later life (Hirokawa, 1998).

[0009] The impact of thymus atrophy is reflected in the periphery, with reduced thymic input to the T cell pool resulting in a less diverse T cell receptor (TCR) repertoire. Altered cytokine profile (Hobbs *et al.*, 1993; Kurashima *et al.*, 1995), changes in CD4<sup>+</sup> and 25 CD8<sup>+</sup> subsets and a bias towards memory as opposed to naïve T cells (Mackall *et al.*, 1995) are also observed. Furthermore, the efficiency of thymopoiesis is impaired with age such that the ability of the immune system to regenerate normal T-cell numbers after T-cell depletion is

eventually lost (Mackall *et al.*, 1995). However, recent work by Douek *et al.* (1998), has shown presumably thymic output to occur even in old age in humans. Excisional DNA products of TCR gene-rearrangement were used to demonstrate circulating, *de novo* produced naïve T cells after HIV infection in older patients. The rate of this output and subsequent 5 peripheral T cell pool regeneration needs to be further addressed since patients who have undergone chemotherapy show a greatly reduced rate of regeneration of the T cell pool, particularly CD4<sup>+</sup> T cells, in post-pubertal patients compared to those who were pre-pubertal (Mackall *et al.*, 1995). This is further exemplified in recent work by Timm and Thoman (1999), who have shown that although CD4<sup>+</sup> T cells are regenerated in old mice post bone 10 marrow transplant (BMT), they appear to show a bias towards memory cells due to the aged peripheral microenvironment, coupled to poor thymic production of naïve T cells.

**[0010]** The thymus essentially consists of developing thymocytes interspersed within the diverse stromal cells (predominantly epithelial cell subsets) which constitute the microenvironment and provide the growth factors and cellular interactions necessary for the 15 optimal development of the T cells. The symbiotic developmental relationship between thymocytes and the epithelial subsets that controls their differentiation and maturation (Boyd *et al.*, 1993), means sex-steroid inhibition could occur at the level of either cell type which would then influence the status of the other. It is less likely that there is an inherent defect within the thymocytes themselves since previous studies, utilizing radiation chimeras, have 20 shown that bone marrow (BM) stem cells are not affected by age (Hirokawa, 1998; Mackall and Gress, 1997) and have a similar degree of thymus repopulation potential as young BM cells. Furthermore, thymocytes in older aged animals retain their ability to differentiate to at least some degree (Mackall and Gress, 1997; George and Ritter, 1996; Hirokawa *et al.*, 1994). However, recent work by Aspinall (1997), has shown a defect within the precursor CD3-CD4- 25 CD8- triple negative (TN) population occurring at the stage of TCRγ chain gene-rearrangement.

#### SUMMARY OF THE INVENTION

**[0011]** The present disclosure teaches methods for preventing illness in a patient by causing the patient's thymus to reactivate and the functional status of the peripheral T cells to 30 be improved, becoming better able to defend against infection or immune system challenge. As used herein, "prevention" and "preventing" refer to complete as well as partial protection of the patient from disease caused by an infectious agent. Optional gene therapy utilizing

genetically modified HSC, lymphoid progenitor, myeloid progenitor or epithelial stem cells, or combinations thereof (the group and each member herein referred to as "GM cells"), can be delivered to a reactivating thymus to create particular immunities. In a preferred embodiment the atrophic thymus in an aged (post-pubertal) patient is reactivated. This reactivated thymus

5 becomes capable of taking up HSC and bone marrow cells from the blood and converting them in the thymus to both new T cells and DC.

**[0012]** In one aspect the present disclosure provides a method for preventing or diminishing the risk of disease in a patient, the method comprising disrupting sex steroid mediated signaling to the thymus in the patient.

10 **[0013]** In one embodiment, bone marrow or HSC are also transplanted into the patient.

**[0014]** In one embodiment the disease is one that has a defined genetic basis.

15 **[0015]** In a preferred embodiment the disease is a T cell disorder selected from the group consisting of viral infections such as by human immunodeficiency virus (HIV), T cell functional disorders, and any other disease or condition that reduces T cells numerically or functionally, directly or indirectly.

20 **[0016]** In another aspect, the present disclosure provides methods for preventing infection by an infectious agent. GM cells that have been genetically modified to resist or prevent infection, activity, replication, and the like, and combinations thereof, of the infectious agent are injected into a patient concurrently with thymic reactivation.

25 **[0017]** In a preferred embodiment, HSC are genetically modified to create resistance to HIV in the T cells formed during and after thymic reactivation. For example, the HSC are modified to include a gene whose product will interfere with HIV infection, function and/or replication in the T cell. This confers a degree of resistance to the virus, thereby preventing disease caused by the virus.

30 **[0018]** In another aspect the present disclosure provides for the reactivation of the thymus by disrupting sex steroid mediated signaling. In one embodiment castration is used to disrupt the sex steroid mediated signaling. In a preferred embodiment chemical castration is used. In another embodiment surgical castration is used. Castration reverses the state of the thymus to its pre-pubertal state, thereby reactivating it.

**[0019]** In a particular embodiment sex steroid mediated signaling to the thymus is blocked by the administration of agonists or antagonists of LHRH, anti-estrogen antibodies,

anti-androgen antibodies, passive (antibody) or active (antigen) anti-LHRH vaccinations, or combinations thereof ("blockers").

**[0020]** In a preferred embodiment, the blocker(s) is administered by a sustained peptide-release formulation. Examples of sustained peptide-release formulations are provided in WO 98/08533, the entire contents of which are incorporated herein by reference.

**[0021]** In the invention, genetically modified HSC are transplanted into the patient, in a preferred embodiment just before, at the time of, or soon after reactivation of the thymus, creating a new population of genetically modified T cells.

#### DESCRIPTION OF THE FIGURES

**[0022]** Figure 1 A and B: Changes in thymocyte number pre- and post-castration. Thymus atrophy results in a significant decrease in thymocyte numbers with age. By 2 weeks post-castration, cell numbers have increased to young adult levels. By 3 weeks post-castration, numbers have significantly increased from the young adult and they are stabilized by 4 weeks post-castration. \*\*\*=Significantly different from young adult (2 month) thymus,  $p<0.001$

**[0023]** Figure 2 A-C: (A) Spleen numbers remain constant with age and post-castration. The B:T cell ratio in the periphery also remains constant (B), however, the CD4:CD8 ratio decreases significantly ( $p<0.001$ ) with age and is restored to normal young levels by 4 weeks post-castration.

**[0024]** Figure 3: Fluorescence Activated Cell Sorter (FACS) profiles of CD4 vs. CD8 thymocyte populations with age and post-castration. Percentages for each quadrant are given above each plot. Subpopulations of thymocytes remain constant with age and there is a synchronous expansion of thymocytes following castration.

**[0025]** Figure 4: Proliferation of thymocytes as detected by incorporation of a pulse of BrdU. Proportion of proliferating thymocytes remains constant with age and following castration.

**[0026]** Figure 5 A-D: Effects of age and castration on proliferation of thymocyte subsets. (A) Proportion of each subset that constitutes the total proliferating population—The proportion of CD8+ T cells within the proliferating population is significantly increased. (B) Percentage of each subpopulation that is proliferating—The TN and CD8 Subsets have significantly less proliferation at 2 years than at 2 months. At 2 weeks post-castration, the TN population has returned to normal young levels of proliferation while the CD8 population

shows a significant increase in proliferation. The level is equivalent to the normal young by 4 weeks post-castration. (C) Overall TN proliferation remains constant with age and post-castration. However (D) the significant decrease in proliferation of the TN1 subpopulation with age is not returned to normal levels by 4 weeks post-castration. \*\*\*=Highly significant,

5 p<0.001, \*\*=significant, p<0.01

[0027] Figure 6: Mice were injected intrathymically with FITC. The number of FITC+ cells in the periphery were calculated 24 hours later. Although the proportion of recent thymic migrants (RTE) remained consistently about 1% of thymus cell number age but was significantly reduced at 2 weeks post-castration, there was a significant (p<0.01) decrease 10 in the RTE cell numbers with age. Following castration, these values were increasing although still significantly lower than young mice at 2 weeks post-castration. With age, a significant increase in the ratio of CD4+ to CD8+ RTE was seen and this was normalized by 1 week post-castration.

[0028] Figure 7 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell 15 numbers following treatment with cyclophosphamide, a chemotherapy agent. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate (cyclophosphamide alone) group at 1 and 2 weeks post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. By 4 weeks, cell numbers are normalized. (n = 3-4 per 20 treatment group and time point).

[0029] Figure 8 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following irradiation (625 Rads) one week after surgical castration. Note the rapid 25 expansion of the thymus in castrated animals when compared to the non-castrate (irradiation alone) group at 1 and 2 weeks post-treatment. (n = 3-4 per treatment group and time point).

[0030] Figure 9 A-C: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following irradiation and castration on the same day. Note the rapid expansion of 30 the thymus in castrated animals when compared to the non-castrate group at 2 weeks post-treatment. However, the difference observed is not as obvious as when mice were castrated 1 week prior to treatment (Fig. 7). (n = 3-4 per treatment group and time point).

[0031] Figure 10: Changes in thymus (A), spleen (B) and lymph node (C) cell numbers following treatment with cyclophosphamide, a chemotherapy agent, and surgical or chemical castration performed on the same day. Note the rapid expansion of the thymus in

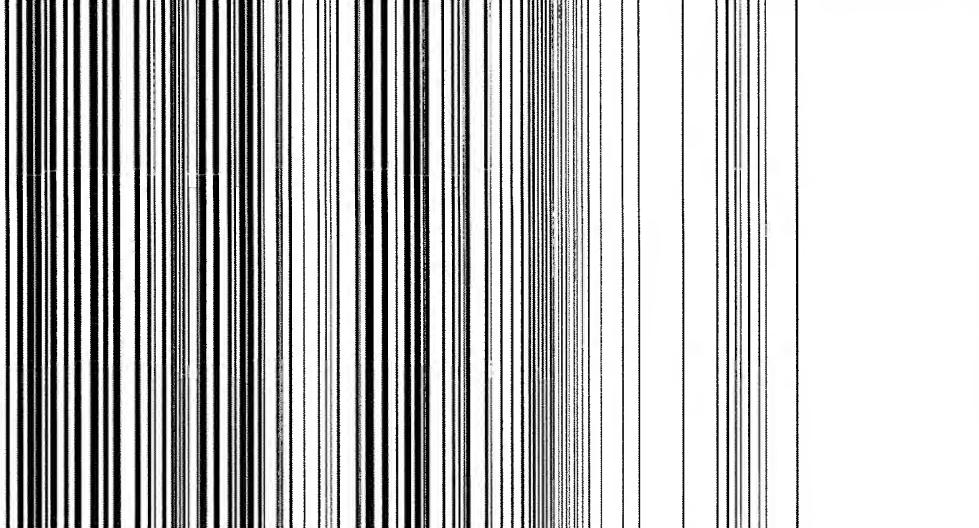
castrated animals when compared to the non-castrate (cyclophosphamide alone) group at 1 and 2 weeks post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. (n = 3-4 per treatment group and time point). Chemical castration is comparable to surgical castration in 5 regeneration of the immune system post-cyclophosphamide treatment.

**[0032]** Figure 11: Lymph node cellularity following foot-pad immunization with Herpes Simplex Virus-1 (HSV-1). Note the increased cellularity in the aged post-castration as compared to the aged non-castrated group. Bottom graph illustrates the overall activated cell number as gated on CD25 vs. CD8 cells by FACS.

10 **[0033]** Figure 12 A-C: V $\beta$ 10 expression on CTL (cytotoxic T lymphocytes) in activated LN (lymph nodes) following HSV-1 inoculation. Note the diminution of a clonal response in aged mice and the reinstatement of the expected response post-castration.

15 **[0034]** Figure 13 A-C: Castration restores responsiveness to HSV-1 immunization. (a) Aged mice showed a significant reduction in total lymph node cellularity post-infection when compared to both the young and post-castrate mice. (b) Representative FACS profiles of activated (CD8 $^+$ CD25 $^+$ ) cells in the LN of HSV-1 infected mice. No difference was seen in proportions of activated CTL with age or post-castration. (c) The decreased cellularity within the lymph nodes of aged mice was reflected by a significant decrease in activated CTL numbers. Castration of the aged mice restored the immune response to HSV-1 with CTL 20 numbers equivalent to young mice. Results are expressed as mean  $\pm$  1 SD of 8-12 mice. \*\* = p  $\leq$  0.01 compared to young (2-month) mice; ^ = p  $\leq$  0.01 compared to aged (non-cx) mice.

25 **[0035]** Figure 14: Popliteal lymph nodes were removed from mice immunized with HSV-1 and cultured for 3 days. CTL assays were performed with non-immunized mice as control for background levels of lysis (as determined by  $^{51}$ Cr-release). Results are expressed



V $\beta$ 10 or V $\beta$ 8.1 is shown as mean  $\pm$ 1SD for 8 mice per group. No difference was observed with age or post-castration. (b) A decrease in CD4/CD8 ratio in the resting LN population was seen with age. This was restored post-castration. Results are expressed as mean $\pm$ 1SD of 8 mice per group. \*\*\* = p $\leq$  0.001 compared to young and castrate mice.

5 [0037] Figure 16 A-D: Changes in thymus (A), spleen (B), lymph node (C) and bone marrow (D) cell numbers following bone marrow transplantation of Ly5 congenic mice. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate group at all time points post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. (n = 3-4 per treatment group and time point). Castrated mice had significantly increased congenic (Ly5.2) cells compared to non-castrated animals (data not shown).

10 [0038] Figure 17 A and B: Changes in thymus cell number in castrated and noncastrated mice after fetal liver reconstitution. (n = 3-4 for each test group.) (A) At two weeks, thymus cell number of castrated mice was at normal levels and significantly higher than that of noncastrated mice (\*p $\leq$  0.05). Hypertrophy was observed in thymuses of castrated mice after four weeks. Noncastrated cell numbers remain below control levels. (B) CD45.2 $^{+}$  cells - CD45.2 $^{+}$  is a marker showing donor derivation. Two weeks after reconstitution donor-derived cells were present in both castrated and noncastrated mice. Four weeks after treatment approximately 85% of cells in the castrated thymus were donor-derived.

15 20 There were no donor-derived cells in the noncastrated thymus.

[0039] Figure 18: FACS profiles of CD4 versus CD8 donor derived thymocyte populations after lethal irradiation and fetal liver reconstitution, followed by surgical castration. Percentages for each quadrant are given to the right of each plot. The age matched control profile is of an eight month old Ly5.1 congenic mouse thymus. Those of castrated and noncastrated mice are gated on CD45.2 $^{+}$  cells, showing only donor derived cells. Two weeks after reconstitution subpopulations of thymocytes do not differ between castrated and noncastrated mice.

[0040] Figure 19 A and B: Myeloid and lymphoid dendritic cell (DC) number after lethal irradiation, fetal liver reconstitution and castration. (n= 3-4 mice for each test group.) 30 Control (striped) bars on the following graphs are based on the normal number of dendritic cells found in untreated age matched mice. (A) Donor-derived myeloid dendritic cells—Two weeks after reconstitution DC were present at normal levels in noncastrated mice. There were

significantly more DC in castrated mice at the same time point. (\*p≤ 0.05). At four weeks DC number remained above control levels in castrated mice. (B) Donor-derived lymphoid dendritic cells—Two weeks after reconstitution DC numbers in castrated mice were double those of noncastrated mice. Four weeks after treatment DC numbers remained above control 5 levels.

**[0041]** Figure 20 A and B: Changes in total and CD45.2<sup>+</sup> bone marrow cell numbers in castrated and noncastrated mice after fetal liver reconstitution. n=3-4 mice for each test group. (A) Total cell number—Two weeks after reconstitution bone marrow cell numbers had normalized and there was no significant difference in cell number between castrated and 10 noncastrated mice. Four weeks after reconstitution there was a significant difference in cell number between castrated and noncastrated mice (\*p≤ 0.05). (B) CD45.2<sup>+</sup> cell number. There was no significant difference between castrated and noncastrated mice with respect to CD45.2<sup>+</sup> cell number in the bone marrow two weeks after reconstitution. CD45.2<sup>+</sup> cell number remained high in castrated mice at four weeks. There were no donor-derived cells in 15 the noncastrated mice at the same time point.

**[0042]** Figure 21 A-C: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in bone marrow of castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (striped) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age 20 matched mice. (A) T cell number—Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells—Two weeks after reconstitution DC cell numbers were normal in both castrated and noncastrated mice. At this time point there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells—Numbers 25 were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

**[0043]** Figure 22 A and B: Change in total and donor (CD45.2<sup>+</sup>) spleen cell numbers in castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) (A) Total cell number—Two weeks after reconstitution cell numbers were decreased 30 and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution cell numbers were approaching normal levels in castrated mice. (B) CD45.2<sup>+</sup> cell number—There was no significant difference between

castrated and noncastrated mice with respect to CD45.2<sup>+</sup> cell number in the spleen, two weeks after reconstitution. CD45.2<sup>+</sup> cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same time point.

5 [0044] Figure 23 A-C: Splenic T cells and myeloid and lymphoid derived dendritic cells (DC) after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (striped) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number—Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived (CD45.2<sup>+</sup>) myeloid dendritic cells—two and four weeks after reconstitution DC numbers 10 were normal in both castrated and noncastrated mice. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived (CD45.2<sup>+</sup>) lymphoid dendritic cells—numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

15 [0045] Figure 24 A and B: Changes in total and donor (CD45.2<sup>+</sup>) lymph node cell numbers in castrated and noncastrated mice after fetal liver reconstitution. (n=3-4 for each test group.) (A) Total cell numbers—Two weeks after reconstitution cell numbers were at normal levels and there was no significant difference between castrated and noncastrated mice. Four weeks after reconstitution cell numbers in castrated mice were at normal levels. 20 (B) CD45.2<sup>+</sup> cell number—There was no significant difference between castrated and noncastrated mice with respect to donor CD45.2<sup>+</sup> cell number in the lymph node two weeks after reconstitution. CD45.2 cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same point.

25 [0046] Figure 25 A-C: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in the mesenteric lymph nodes of castrated and non-castrated mice after fetal liver reconstitution. (n=3-4 mice for each test group.) Control (striped) bars are the number of T cells and dendritic cells found in untreated age matched mice. (A) T cell numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells were normal in both castrated 30 and noncastrated mice. At four weeks they were decreased. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells—Numbers were at normal levels two and four weeks after

reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

**[0047]** Figure 26: The phenotypic composition of peripheral blood lymphocytes was analyzed in human patients (all >60 years) undergoing LHRH agonist treatment for prostate cancer. Patient samples were analyzed before treatment and 4 months after beginning LHRH agonist treatment. Total lymphocyte cell numbers per ml of blood were at the lower end of control values before treatment in all patients. Following treatment, 6/9 patients showed substantial increases in total lymphocyte counts (in some cases a doubling of total cells was observed). Correlating with this was an increase in total T cell numbers in 6/9 patients.

10 Within the CD4<sup>+</sup> subset, this increase was even more pronounced with 8/9 patients demonstrating increased levels of CD4 T cells. A less distinctive trend was seen within the CD8<sup>+</sup> subset with 4/9 patients showing increased levels, albeit generally to a smaller extent than CD4<sup>+</sup> T cells.

**[0048]** Figure 27: Analysis of human patient blood before and after LHRH-agonist treatment demonstrated no substantial changes in the overall proportion of T cells, CD4 or CD8 T cells, and a variable change in the CD4:CD8 ratio following treatment. This indicates the minimal effect of treatment on the homeostatic maintenance of T cell subsets despite the substantial increase in overall T cell numbers following treatment. All values were comparative to control values.

20 **[0049]** Figure 28: Analysis of the proportions of B cells and myeloid cells (NK, NKT and macrophages) within the peripheral blood of human patients undergoing LHRH agonist treatment demonstrated a varying degree of change within subsets. While NK, NKT and macrophage proportions remained relatively constant following treatment, the proportion of B cells was decreased in 4/9 patients.

25 **[0050]** Figure 29: Analysis of the total cell numbers of B and myeloid cells within the peripheral blood of human patients post-treatment showed clearly increased levels of NK (5/9 patients), NKT (4/9 patients) and macrophage (3/9 patients) cell numbers post-treatment. B cell numbers showed no distinct trend with 2/9 patients showing increased levels; 4/9 patients showing no change and 3/9 patients showing decreased levels.

30 **[0051]** Figure 30 A and B: The major change seen post-LHRH agonist treatment was within the T cell population of the peripheral blood. In particular there was a selective increase in the proportion of naïve (CD45RA<sup>+</sup>) CD4+ cells, with the ratio of naïve

(CD45RA<sup>+</sup>) to memory (CD45RO<sup>+</sup>) in the CD4<sup>+</sup> T cell subset increasing in 6/9 of the human patients.

**[0052]** Figure 31: Decrease in the impedance of skin using various laser pulse energies. There is a decrease in skin impedance in skin irradiated at energies as low as 10 mJ, using the fitted curve to interpolate data.

**[0053]** Figure 32: Permeation of a pharmaceutical through skin. Permeability of the skin, using insulin as a sample pharmaceutical, was greatly increased through laser irradiation.

**[0054]** Figure 33: Change in fluorescence of skin over time after the addition of 5-aminolevulenic acid (ALA) and a single impulse transient to the skin. The peak of intensity occurs at about 640 nm and is highest after 210 minutes (dashed line) post-treatment.

**[0055]** Figure 34: Change in fluorescence of skin over time after the addition of 5-aminolevulenic acid (ALA) without an impulse transient. There is little change in the intensity at different time points.

**[0056]** Figure 35: Comparison of change in fluorescence of skin after the addition of 5-aminolevulenic acid (ALA) and a single impulse transient under various peak stresses. The degree of permeabilization of the stratum corneum depends on the peak stress.

#### DETAILED DESCRIPTION OF THE INVENTION

**[0057]** The present disclosure comprises methods for preventing disease in a patient. As described above, the aged (post-pubertal) thymus causes the body's immune system to function at less than peak levels. The present disclosure uses reactivation of the thymus to improve immune system function, thereby increasing resistance to, and preventing infection by, various foreign agents. In some instances prevention of infection is not achieved, but in such cases, a stronger, more resilient immune system will aid the body in decreasing the extent and length of infection.

**[0058]** In some instances, the methods of the invention use genetically modified hematopoietic stem cells, lymphoid progenitor cells, myeloid progenitor cells, epithelial stem cells or combinations thereof (GM cells) to produce an immune system resistant to attack by particular antigens. This embodiment is described in co-pending United States patent application 09/758,910. An appropriate gene or polynucleotide that will create or induce resistance to one or more infectious agents is engineered into the stem and/or progenitor cells. The modified cells are injected into a patient whose thymus is being reactivated by the methods of this invention. The modified stem and progenitor cells are taken up by the thymus

and converted into T cells, dendritic cells, and other cells produced in the thymus. Each of these new cells contains the genetic modification of the parent stem/progenitor cell, and is thereby resistant to infection by the agent or agents. B cells are also increased in number in the bone marrow, blood and peripheral lymphoid organs, such as the spleen and lymph nodes, 5 within two weeks of castration.

**[0059]** The recipient's thymus may be reactivated by disruption of sex steroid mediated signaling to the thymus. This disruption reverses the hormonal status of the recipient. A preferred method for creating disruption is through castration. Methods for castration include but are not limited to chemical castration and surgical castration. During or 10 after the castration step, GM cells are transplanted into the patient. These cells are accepted by the thymus as belonging to the patient and become part of the production of new T cells and DC by the thymus. The resulting population of T cells contain the genetic modifications that had been inserted into the stem/progenitor cells.

**[0060]** A preferred method of reactivating the thymus is by blocking the direct and/or 15 indirect stimulatory effects of LHRH on the pituitary, which leads to a loss of the gonadotrophins FSH and LH. These gonadotrophins normally act on the gonads to release sex hormones, in particular estrogens in females and testosterone in males; the release is blocked by the loss of FSH and LH. The direct consequences of this are an immediate drop in the plasma levels of sex steroids, and as a result, progressive release of the inhibitory signals 20 on the thymus. The degree and kinetics of thymic regrowth can be enhanced by injection of CD34<sup>+</sup> hematopoietic cells (ideally autologous).

**[0061]** This invention may be used with any animal species (including humans) having sex steroid driven maturation and an immune system, such as mammals and marsupials, preferably large mammals, and most preferably humans.

**[0062]** The terms "regeneration," "reactivation" and "reconstitution" and their 25 derivatives are used interchangeably herein, and refer to the recovery of an atrophied thymus to its active state.

**[0063]** "Castration," as used herein, means the marked reduction or elimination of sex 30 steroid production and distribution in the body. This effectively returns the patient to pre-pubescent status when the thymus is fully functioning. Surgical castration removes the patient's gonads.

**[0064]** A less permanent version of castration is through the administration of a chemical for a period of time, referred to herein as "chemical castration." A variety of chemicals are capable of functioning in this manner. During the chemical delivery, and for a period of time afterwards, the patient's hormone production is turned off. Preferably the castration is reversed upon termination of chemical delivery.

5 **DISRUPTION OF SEX STEROID MEDIATED SIGNALING TO THE THYMUS**

**[0065]** As will be readily understood, sex steroid mediated signaling to the thymus can be disrupted in a range of ways well known to those of skill in the art, some of which are described herein. For example, inhibition of sex steroid production or blocking of one or 10 more sex steroid receptors within the thymus will accomplish the desired disruption, as will administration of sex steroid agonists or antagonists, or active (antigen) or passive (antibody) anti-sex steroid vaccinations. Inhibition of sex steroid production can also be achieved by administration of one or more sex steroid analogs. In some clinical cases, permanent removal of the gonads via physical castration may be appropriate.

15 **[0066]** In a preferred embodiment, the sex steroid mediated signaling to the thymus is disrupted by administration of a sex steroid analog, preferably an analog of luteinizing hormone-releasing hormone (LHRH). Sex steroid analogs and their use in therapies and chemical castration are well known. Such analogs include, but are not limited to, the following agonists of the LHRH receptor (LHRH-R): Buserelin (Hoechst), Cystorelin

20 (Hoechst), Decapeptyl (trade name Debiopharm; Ipsen/Beaufour), Deslorelin (Balance Pharmaceuticals), Gonadorelin (Ayerst), Goserelin (trade name Zoladex; Zeneca), Histrelin (Ortho), Leuprolide (trade name Lupron; Abbott/TAP), Leuprorelin (Plosker et al.), Lutrelin (Wyeth), Meterelin (WO9118016), Nafarelin (Syntex), and Triptorelin (U.S. Patent No.

4,010,125). LHRH analogs also include, but are not limited to, the following antagonists of 25 the LHRH-R: Abarelix (trade name Plenaxis; Praecis) and Cetrorelix (trade name; Zentaris). Combinations of agonists, combinations of antagonists, and combinations of agonists and antagonists are also included. The disclosures of each the references referred to above are incorporated herein by reference. It is currently preferred that the analog is Deslorelin (described in U.S. Patent No. 4,218,439). For a more extensive list, see Vickery et al., 1984.

30 **[0067]** In a preferred embodiment, an LHRH-R antagonist is delivered to the patient, followed by an LHRH-R agonist. This protocol abolishes or limits any spike of sex steroid production, before the decrease in sex steroid production, that might be produced by the

administration of the agonist. In an alternate embodiment, an LHRH-R agonist that creates little or no sex steroid production spike is used, with or without the prior administration of an LHRH-R antagonist.

**[0068]** While the stimulus for thymic reactivation is fundamentally based on the inhibition of the effects of sex steroids and/or the direct effects of the LHRH analogs, it may be useful to include additional substances which can act in concert to enhance the thymic effect. Such compounds include but are not limited to Interleukin 2 (IL2), Interleukin 7 (IL7), Interleukin 15 (IL15), members of the epithelial and fibroblast growth factor families, Stem Cell Factor, granulocyte colony stimulating factor (GCSF) and keratinocyte growth factor (KGF). It is envisaged that these additional compound(s) would only be given once at the initial LHRH analog application. However, additional doses of any one or combination of these substances may be given at any time to further stimulate the thymus. In addition, steroid receptor based modulators, which may be targeted to be thymic specific, may be developed and used.

**[0069]** An important feature of the thymic improvement and that of the immune system in general is that following disruption of the sex steroids, there is also an enhancement of bone marrow function. This includes, but is not limited to, increased production and/or release of HSC. These HSC are able to migrate to the thymus and assist in the increased thymopoiesis.

## 20 PHARMACEUTICAL COMPOSITIONS

**[0070]** The compounds used in this invention can be supplied in any pharmaceutically acceptable carrier or without a carrier. Examples include physiologically compatible coatings, solvents and diluents. For parenteral, subcutaneous, intravenous and intramuscular administration, the compositions may be protected such as by encapsulation. Alternatively, the compositions may be provided with carriers that protect the active ingredient(s), while allowing a slow release of those ingredients. Numerous polymers and copolymers are known in the art for preparing time-release preparations, such as various versions of lactic acid/glycolic acid copolymers. See, for example, U.S. Patent No. 5,410,016, which uses modified polymers of polyethylene glycol (PEG) as a biodegradeable coating.

**[0071]** Formulations intended to be delivered orally can be prepared as liquids, capsules, tablets, and the like. These compositions can include, for example, excipients,

diluents, and/or coverings that protect the active ingredient(s) from decomposition. Such formulations are well known.

**[0072]** In any of the formulations, other compounds that do not negatively affect the activity of the LHRH analogs may be included. Examples are various growth factors and 5 other cytokines as described herein.

#### DOSE

**[0073]** The LHRH analog can be administered in a one-time dose that will last for a period of time. Preferably, the formulation will be effective for one to two months, more preferably for one to three months. Reduction of sex steroids in the blood to minimal values 10 takes approximately 3 weeks when using an agonist and 1 day when using an antagonist. In a preferred embodiment, a dose is prepared to last as long as a periodic epidemic lasts. For example, "flu season" occurs usually during the winter months. A formulation of an LHRH analog can be made and delivered as described herein to protect a patient for a period of two or more months starting at the beginning of the flu season, with additional doses delivered 15 every two or more months until the risk of infection decreases or disappears.

**[0074]** The standard dose varies with type of analog used. In general, the dose is between about 0.01  $\mu$ g/kg and about 10 mg/kg, preferably between about 0.01 mg/kg and about 5 mg/kg. Dose varies with the LHRH analog or vaccine used. For example, for males, under current dosing conditions, 10.8 mg goserelin as a single dose (effective for 3 months) is 20 administered on day-21 (before date of thymic enhancement), with a further injection of 3.6 mg on day 63 (after date of thymic enhancement), which will be effective for another 28 days or so. For females, 3/6 mg (effective for 28 days) is administered on day --1, 7, 35 and 63.

**[0075]** The formulation can be made to enhance the immune system. Alternatively, the formulation can be prepared to specifically deter infection by flu viruses while enhancing 25 the immune system. This latter formulation would include GM cells that have been engineered to create resistance to flu viruses (see below). The GM cells can be administered with the LHRH analog formulation or separately, both spatially and/or in time. As with the non-GM cells, multiple doses over time can be administered to a patient to create protection and prevent infection with the flu virus over the length of the flu season.

#### 30 DELIVERY OF AGENTS FOR CHEMICAL CASTRATION

**[0076]** Delivery of the compounds of this invention can be accomplished via a number of methods known to persons skilled in the art. One standard procedure for administering

chemical inhibitors to inhibit sex steroid mediated signaling to the thymus utilizes a single dose of an LHRH agonist that is effective for three months. For this a simple one-time i.v. or i.m. injection would not be sufficient as the agonist would be cleared from the patient's body well before the three months are over. Instead, a depot injection or an implant may be used, 5 or any other means of delivery of the inhibitor that will allow slow release of the inhibitor. Likewise, a method for increasing the half life of the inhibitor within the body, such as by modification of the chemical, while retaining the function required herein, may be used.

**[0077]** Examples of more useful delivery mechanisms include, but are not limited to, laser irradiation of the skin, and creation of high pressure impulse transients (also called stress 10 waves or impulse transients) on the skin, each method accompanied or followed by placement of the compound(s) with or without carrier at the same locus. A preferred method of this placement is in a patch placed and maintained on the skin for the duration of the treatment.

**[0078]** One means of delivery utilizes a laser beam, specifically focused, and lasing at an appropriate wavelength, to create small perforations or alterations in the skin of a patient.

15 See U.S. Pat. No. 4,775,361, U.S. Pat. No. 5,643,252, U.S. Pat. No. 5,839,446, and U.S. Pat. No. 6,056,738, all of which are incorporated herein by reference. In a preferred embodiment, the laser beam has a wavelength between 0.2 and 10 microns. More preferably, the wavelength is between about 1.5 and 3.0 microns. Most preferably the wavelength is about 2.94 microns. In one embodiment, the laser beam is focused with a lens to produce an 20 irradiation spot on the skin through the epidermis of the skin. In an additional embodiment, the laser beam is focused to create an irradiation spot only through the stratum corneum of the skin.

**[0079]** As used herein, "ablation" and "perforation" mean a hole created in the skin. Such a hole can vary in depth; for example it may only penetrate the stratum corneum, it may 25 penetrate all the way into the capillary layer of the skin, or it may terminate anywhere in between. As used herein, "alteration" means a change in the skin structure, without the creation of a hole, that increases the permeability of the skin. As with perforation, skin can be altered to any depth.

**[0080]** Several factors may be considered in defining the laser beam, including 30 wavelength, energy fluence, pulse temporal width and irradiation spot-size. In a preferred embodiment, the energy fluence is in the range of 0.03-100,000 J/cm<sup>2</sup>. More preferably, the energy fluence is in the range of 0.03 - 9.6 J/cm<sup>2</sup>. The beam wavelength is dependent in part

on the laser material, such as Er:YAG. The pulse temporal width is a consequence of the pulse width produced by, for example, a bank of capacitors, the flashlamp, and the laser rod material. The pulse width is optimally between 1 fs (femtosecond) and 1,000  $\mu$ s.

**[0081]** According to this method the perforation or alteration produced by the laser 5 need not be produced with a single pulse from the laser. In a preferred embodiment a perforation or alteration through the stratum corneum is produced by using multiple laser pulses, each of which perforates or alters only a fraction of the target tissue thickness.

**[0082]** To this end, one can roughly estimate the energy required to perforate or alter 10 the stratum corneum with multiple pulses by taking the energy in a single pulse and dividing by the number of pulses desirable. For example, if a spot of a particular size requires 1 J of 15 energy to produce a perforation or alteration through the entire stratum corneum, then one can produce qualitatively similar perforation or alteration using ten pulses, each having 1/10th the energy. Because it is desirable that the patient not move the target tissue during the irradiation (human reaction times are on the order of 100 ms or so), and that the heat produced during each pulse not significantly diffuse, in a preferred embodiment the pulse repetition rate from the laser should be such that complete perforation is produced in a time of less than 100 ms. Alternatively, the orientation of the target tissue and the laser can be mechanically fixed so that changes in the target location do not occur during the longer irradiation time.

**[0083]** To penetrate the skin in a manner that induces little or no blood flow, skin can 20 be perforated or altered through the outer surface, such as the stratum corneum layer, but not as deep as the capillary layer. The laser beam is focussed precisely on the skin, creating a beam diameter at the skin in the range of approximately 0.5 microns - 5.0 cm. Optionally, the spot can be slit-shaped, with a width of about 0.05-0.5 mm and a length of up to 2.5 mm. The width can be of any size, being controlled by the anatomy of the area irradiated and the 25 desired permeation rate of the fluid to be removed or the pharmaceutical to be applied. The focal length of the focusing lens can be of any length, but in one embodiment it is 30 mm.

**[0084]** By modifying wavelength, pulse length, energy fluence (which is a function of 30 the laser energy output (in Joules) and size of the beam at the focal point ( $\text{cm}^2$ )), and irradiation spot size, it is possible to vary the effect on the stratum corneum between ablation (perforation) and non-ablative modification (alteration). Both ablation and non-ablative alteration of the stratum corneum result in enhanced permeation of subsequently applied pharmaceuticals.

**[0085]** For example, by reducing the pulse energy while holding other variables constant, it is possible to change between ablative and non-ablative tissue-effect. Using an Er:YAG laser having a pulse length of about 300  $\mu$ s, with a single pulse or radiant energy and irradiating a 2 mm spot on the skin, a pulse energy above approximately 100 mJ causes partial or complete ablation, while any pulse energy below approximately 100 mJ causes partial ablation or non-ablative alteration to the stratum corneum. Optionally, by using multiple pulses, the threshold pulse energy required to enhance permeation of body fluids or for pharmaceutical delivery is reduced by a factor approximately equal to the number of pulses.

**[0086]** Alternatively, by reducing the spot size while holding other variables constant, it is also possible to change between ablative and non-ablative tissue-effect. For example, halving the spot area will result in halving the energy required to produce the same effect. Irradiation down to 0.5 microns can be obtained, for example, by coupling the radiant output of the laser into the objective lens of a microscope objective. (e.g., as available from Nikon, Inc., Melville, NY). In such a case, it is possible to focus the beam down to spots on the order of the limit of resolution of the microscope, which is perhaps on the order of about 0.5 microns. In fact, if the beam profile is Gaussian, the size of the affected irradiated area can be less than the measured beam size and can exceed the imaging resolution of the microscope. To non-ablatively alter tissue in this case, it would be suitable to use a  $3.2 \text{ J/cm}^2$  energy fluence, which for a half-micron spot size would require a pulse energy of about 5 nJ. This low a pulse energy is readily available from diode lasers, and can also be obtained from, for example, the Er:YAG laser by attenuating the beam by an absorbing filter, such as glass.

**[0087]** Optionally, by changing the wavelength of radiant energy while holding the other variables constant, it is possible to change between an ablative and non-ablative tissue-effect. For example, using Ho:YAG (holmium: YAG; 2.127 microns) in place of the Er:YAG (erbium: YAG; 2.94 microns) laser, would result in less absorption of energy by the tissue, creating less of a perforation or alteration.

**[0088]** Picosecond and femtosecond pulses produced by lasers can also be used to produce alteration or ablation in skin. This can be accomplished with modulated diode or related microchip lasers, which deliver single pulses with temporal widths in the 1 femtosecond to 1 ms range. (See D. Stern *et al.*, "Corneal Ablation by Nanosecond, Picosecond, and Femtosecond Lasers at 532 and 625 nm," *Corneal Laser Ablation*, Vol. 107,

pp. 587-592 (1989), incorporated herein by reference, which discloses the use of pulse lengths down to 1 femtosecond).

**[0089]** Another delivery method uses high pressure impulse transients on skin to create permeability. See U.S. Pat. No. 5,614,502, and U.S. Pat. No. 5,658,892, both of which are incorporated herein by reference. High pressure impulse transients, e.g., stress waves (e.g., laser stress waves (LSW) when generated by a laser), with specific rise times and peak stresses (or pressures), can safely and efficiently effect the transport of compounds, such as those of the present disclosure, through layers of epithelial tissues, such as the stratum corneum and mucosal membranes. These methods can be used to deliver compounds of a wide range of sizes regardless of their net charge. In addition, impulse transients used in the present methods avoid tissue injury.

**[0090]** Prior to exposure to an impulse transient, an epithelial tissue layer, e.g., the stratum corneum, is likely impermeable to a foreign compound; this prevents diffusion of the compound into cells underlying the epithelial layer. Exposure of the epithelial layer to the impulse transients enables the compound to diffuse through the epithelial layer. The rate of diffusion, in general, is dictated by the nature of the impulse transients and the size of the compound to be delivered.

**[0091]** The rate of penetration through specific epithelial tissue layers, such as the stratum corneum of the skin, also depends on several other factors including pH, the metabolism of the cutaneous substrate tissue, pressure differences between the region external to the stratum corneum, and the region internal to the stratum corneum, as well as the anatomical site and physical condition of the skin. In turn, the physical condition of the skin depends on health, age, sex, race, skin care, and history. For example, prior contacts with organic solvents or surfactants affect the physical condition of the skin.

**[0092]** The amount of compound delivered through the epithelial tissue layer will also depend on the length of time the epithelial layer remains permeable, and the size of the surface area of the epithelial layer which is made permeable.

**[0093]** The properties and characteristics of impulse transients are controlled by the energy source used to create them. See WO 98/23325, which is incorporated herein by reference. However, their characteristics are modified by the linear and non-linear properties of the coupling medium through which they propagate. The linear attenuation caused by the coupling medium attenuates predominantly the high frequency components of the impulse

transients. This causes the bandwidth to decrease with a corresponding increase in the rise time of the impulse transient. The non-linear properties of the coupling medium, on the other hand, cause the rise time to decrease. The decrease of the rise time is the result of the dependence of the sound and particle velocity on stress (pressure). As the stress increases, the 5 sound and the particle velocity increase as well. This causes the leading edge of the impulse transient to become steeper. The relative strengths of the linear attenuation, non-linear coefficient, and the peak stress determine how long the wave has to travel for the increase in steepness of rise time to become substantial.

**[0094]** The rise time, magnitude, and duration of the impulse transient are chosen to 10 create a non-destructive (i.e., non-shock wave) impulse transient that temporarily increases the permeability of the epithelial tissue layer. Generally the rise time is at least 1 ns, and is more preferably about 10 ns.

**[0095]** The peak stress or pressure of the impulse transients varies for different 15 epithelial tissue or cell layers. For example, to transport compounds through the stratum corneum, the peak stress or pressure of the impulse transient should be set to at least 400 bar; more preferably at least 1,000 bar, but no more than about 2,000 bar.

**[0096]** For epithelial mucosal layers, the peak pressure should be set to between 300 20 bar and 800 bar, and is preferably between 300 bar and 600 bar. The impulse transients preferably have durations on the order of a few tens of ns, and thus interact with the epithelial tissue for only a short period of time. Following interaction with the impulse transient, the epithelial tissue is not permanently damaged, but remains permeable for up to about three minutes.

**[0097]** In addition, these methods involve the application of only a few discrete high 25 amplitude pulses to the patient. The number of impulse transients administered to the patient is typically less than 100, more preferably less than 50, and most preferably less than 10. When multiple optical pulses are used to generate the impulse transient, the time duration between sequential pulses is 10 to 120 seconds, which is long enough to prevent permanent damage to the epithelial tissue.

**[0098]** Properties of impulse transients can be measured using methods standard in the 30 art. For example, peak stress or pressure, and rise time can be measured using a polyvinylidene fluoride (PVDF) transducer method as described in Doukas *et al.*, *Ultrasound Med. Biol.*, 21:961 (1995).

**[0099]** Impulse transients can be generated by various energy sources. The physical phenomenon responsible for launching the impulse transient is, in general, chosen from three different mechanisms: (1) thermoelastic generation; (2) optical breakdown; or (3) ablation.

**[0100]** For example, the impulse transients can be initiated by applying a high energy 5 laser source to ablate a target material, and the impulse transient is then coupled to an epithelial tissue or cell layer by a coupling medium. The coupling medium can be, for example, a liquid or a gel, as long as it is non-linear. Thus, water, oil such as castor oil, an isotonic medium such as phosphate buffered saline (PBS), or a gel such as a collagenous gel, can be used as the coupling medium.

**[0101]** In addition, the coupling medium can include a surfactant that enhances 10 transport, e.g., by prolonging the period of time in which the stratum corneum remains permeable to the compound following the generation of an impulse transient. The surfactant can be, e.g., ionic detergents or nonionic detergents and thus can include, e.g., sodium lauryl sulfate, cetyl trimethyl ammonium bromide, and lauryl dimethyl amine oxide.

**[0102]** The absorbing target material acts as an optically triggered transducer. 15 Following absorption of light, the target material undergoes rapid thermal expansion, or is ablated, to launch an impulse transient. Typically, metal and polymer films have high absorption coefficients in the visible and ultraviolet spectral regions.

**[0103]** Many types of materials can be used as the target material in conjunction with 20 a laser beam, provided they fully absorb light at the wavelength of the laser used. The target material can be composed of a metal such as aluminum or copper; a plastic, such as polystyrene, e.g., black polystyrene; a ceramic; or a highly concentrated dye solution. The target material must have dimensions larger than the cross-sectional area of the applied laser energy. In addition, the target material must be thicker than the optical penetration depth so 25 that no light strikes the surface of the skin. The target material must also be sufficiently thick to provide mechanical support. When the target material is made of a metal, the typical thickness will be 1/32 to 1/16 inch. For plastic target materials, the thickness will be 1/16 to 1/8 inch.

**[0104]** Impulse transients can also be enhanced using confined ablation. In confined 30 ablation, a laser beam transparent material, such as a quartz optical window, is placed in close contact with the target material. Confinement of the plasma, created by ablating the target material by using the transparent material, increases the coupling coefficient by an order of

magnitude (Fabro *et al.*, *J. Appl. Phys.*, 68:775, 1990). The transparent material can be quartz, glass, or transparent plastic.

**[0105]** Since voids between the target material and the confining transparent material allow the plasma to expand, and thus decrease the momentum imparted to the target, the transparent material is preferably bonded to the target material using an initially liquid adhesive, such as carbon-containing epoxies, to prevent such voids.

**[0106]** The laser beam can be generated by standard optical modulation techniques known in the art, such as by employing Q-switched or mode-locked lasers using, for example, electro- or acousto-optic devices. Standard commercially available lasers that can operate in a pulsed mode in the infrared, visible, and/or infrared spectrum include Nd:YAG, Nd:YLF, CO<sub>2</sub>, excimer, dye, Ti:sapphire, diode, holmium (and other rare-earth materials), and metal-vapor lasers. The pulse widths of these light sources are adjustable, and can vary from several tens of picoseconds (ps) to several hundred microseconds. For use in the present disclosure, the optical pulse width can vary from 100 ps to about 200 ns and is preferably between about 500 ps and 40 ns.

**[0107]** Impulse transients can also be generated by extracorporeal lithotripters (one example is described in Coleman *et al.*, *Ultrasound Med. Biol.*, 15:213-227, 1989). These impulse transients have rise times of 30 to 450 ns, which is longer than laser-generated impulse transients. To form an impulse transient of the appropriate rise time for the new methods using an extracorporeal lithotripter, the impulse transient is propagated in a non-linear coupling medium (e.g., water) for a distance determined by equation (1), above. For example, when using a lithotripter creating an impulse transient having a rise time of 100 ns and a peak pressure of 500 barr, the distance that the impulse transient should travel through the coupling medium before contacting an epithelial cell layer is approximately 5 mm.

**[0108]** An additional advantage of this approach for shaping impulse transients generated by lithotripters is that the tensile component of the wave will be broadened and attenuated as a result of propagating through the non-linear coupling medium. This propagation distance should be adjusted to produce an impulse transient having a tensile component that has a pressure of only about 5 to 10% of the peak pressure of the compressive component of the wave. Thus, the shaped impulse transient will not damage tissue.

**[0109]** The type of lithotripter used is not critical. Either an electrohydraulic, electromagnetic, or piezoelectric lithotripter can be used.

**[0110]** The impulse transients can also be generated using transducers, such as piezoelectric transducers. Preferably, the transducer is in direct contact with the coupling medium, and undergoes rapid displacement following application of an optical, thermal, or electric field to generate the impulse transient. For example, dielectric breakdown can be used, and is typically induced by a high-voltage spark or piezoelectric transducer (similar to those used in certain extracorporeal lithotripters, Coleman *et al.*, *Ultrasound Med. Biol.*, 15:213-227, 1989). In the case of a piezoelectric transducer, the transducer undergoes rapid expansion following application of an electrical field to cause a rapid displacement in the coupling medium.

5 **[0111]** In addition, impulse transients can be generated with the aid of fiber optics. Fiber optic delivery systems are particularly maneuverable and can be used to irradiate target materials located adjacent to epithelial tissue layers to generate impulse transients in hard-to reach places. These types of delivery systems, when optically coupled to lasers, are preferred as they can be integrated into catheters and related flexible devices, and used to irradiate most 10 organs in the human body. In addition, to launch an impulse transient having the desired rise times and peak stress, the wavelength of the optical source can be easily tailored to generate the appropriate absorption in a particular target material.

15 **[0112]** Alternatively, an energetic material can produce an impulse transient in response to a detonating impulse. The detonator can detonate the energetic material by 20 causing an electrical discharge or spark.

25 **[0113]** Hydrostatic pressure can be used in conjunction with impulse transients to enhance the transport of a compound through the epithelial tissue layer. Since the effects induced by the impulse transients last for several minutes, the transport rate of a drug diffusing passively through the epithelial cell layer along its concentration gradient can be increased by applying hydrostatic pressure on the surface of the epithelial tissue layer, e.g., the stratum corneum of the skin, following application of the impulse transient.

#### GENETIC MODIFICATION OF STEM OR PROGENITOR CELLS

##### Genes

30 **[0114]** Useful genes and gene fragments (polynucleotides) for this invention include those that code for resistance to infection of T cells by a particular infectious agent or agents. Such infections agents include, but are not limited to, HIV, T cell leukemia virus, and other viruses that cause lymphoproliferative diseases. With respect to HIV/AIDS, a number of

genes and gene fragments may be used, including, but not limited to, the nef transcription factor; a gene that codes for a ribozyme that specifically cuts HIV genes, such as *tat* and *rev* (Bauer G., *et al.* (1997); the trans-dominant mutant form of HIV-1 *rev* gene, RevM10, which has been shown to inhibit HIV replication (Bonyhadi *et al.* 1997); an overexpression construct 5 of the HIV-1 *rev*-responsive element (RRE) (Kohn *et al.*, 1999); any gene that codes for an RNA or protein whose expression is inhibitory to HIV infection of the cell or replication; and fragments and combinations thereof.

[0115] These genes or gene fragments are used in a stably expressable form. The term "stably expressable form" as used herein means that the product (RNA and/or protein) of the 10 gene or gene fragment ("functional fragment") is capable of being expressed on at least a semi-permanent basis in a host cell after transfer of the gene or gene fragment to that cell, as well as in that cell's progeny after division and/or differentiation. This requires that the gene or gene fragment, whether or not contained in a vector, has appropriate signaling sequences for transcription of the DNA to RNA. Additionally, when a protein coded for by the gene or 15 gene fragment is the active molecule that affects the patient's condition, the DNA will also code for translation signals.

[0116] In most cases the genes or gene fragments will be contained in vectors. Those of ordinary skill in the art are aware of expression vectors that may be used to express the desired RNA or protein. Expression vectors are vectors that are capable of directing 20 transcription of DNA sequences contained therein and translation of the resulting RNA. Expression vectors are capable of replication in the cells to be genetically modified, and include plasmids, bacteriophage, viruses, and minichromosomes. Alternatively the gene or gene fragment may become an integral part of the cell's chromosomal DNA. Recombinant vectors and methodology are in general well-known.

[0117] Expression vectors useful for expressing the proteins of the present disclosure 25 contain an origin of replication. Suitably constructed expression vectors contain an origin of replication for autonomous replication in the cells, or are capable of integrating into the host cell chromosomes. Such vectors may also contain selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. Promoters are 30 DNA sequences that direct RNA polymerase to bind to DNA and initiate RNA synthesis; strong promoters cause such initiation at high frequency. The expression vectors of the present disclosure are operably linked to DNA coding for an RNA or protein to be used in this

invention, *i.e.*, the vectors are capable of directing both replication of the attached DNA molecule and expression of the RNA or protein encoded by the DNA molecule. Thus, for proteins, the expression vector must have an appropriate transcription start signal upstream of the attached DNA molecule, maintaining the correct reading frame to permit expression of the

5 DNA molecule under the control of the control sequences and production of the desired protein encoded by the DNA molecule. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors and specifically designed plasmids or viruses. Preferably, an inducible promoter is used so that the amount and timing of expression of the inserted gene or polynucleotide can be controlled.

10 Cells

[0118] Hematopoietic stem cells (HSC) are the preferred cells for genetic modification. These may be derived from bone marrow, peripheral blood, or umbilical cord, or any other source of HSC, and may be either autologous or nonautologous. Also useful are lymphoid and myeloid progenitor cells and epithelial stem cells, also either autologous or

15 nonautologous.

[0119] In the event that nonautologous (donor) cells are used, tolerance to these cells is created during the step of thymus reactivation. During or after the initiation of blockage of sex steroid mediated signaling to the thymus, the relevant genetically modified donor cells are transplanted into the recipient. These cells are accepted by the thymus as belonging to the

20 recipient and become part of the production of new T cells and DC by the thymus. The resulting population of T cells recognize both the recipient and donor as self, thereby creating tolerance for a graft from the donor. See copending patent application U.S.S.N. 09/\_\_\_\_\_, which is incorporated herein by reference.

Methods of Genetic Modification

25 [0120] Standard recombinant methods can be used to introduce genetic modifications into the cells being used for gene therapy. For example, retroviral vector transduction of cultured HSC is one successful method (Belmont and Jurecic, 1997, Bahnsen, A.B., *et al.*, 1997). Additional vectors include, but are not limited to, those that are adenovirus derived or lentivirus derived, and Moloney murine leukemia virus-derived vectors.

30 [0121] Also useful are the following methods: particle-mediated gene transfer such as with the gene gun (Yang and Ziegelhoffer, 1994), liposome-mediated gene transfer (Nabel *et al.*, 1992), coprecipitation of genetically modified vectors with calcium phosphate (Graham

and Van Der Eb, 1973), electroporation (Potter *et al.*, 1984), and microinjection (Capecci, 1980), as well as any other method that can stably transfer a gene or oligonucleotide, preferably in a vector, into the HSC and other cells to be genetically modified such that the gene will be expressed at least part of the time.

5 PREVENTION

**[0122]** The present disclosure provides methods for preventing, or increasing resistance to, infection of a patient through reactivation of a patient's thymus. This is accomplished through disruption of sex steroid mediated signaling to the thymus. At this stage, the patient's immune system is rejuvenated, increasing its response to foreign antigens 10 such as viruses and bacteria. This is shown, for example, in figures 10-15, which show the effects of thymic reactivation on the mouse immune system, as demonstrated with viral (HSV) challenge. The mice having prior reactivation of the thymus demonstrate resistance to HSV infection, while those not having thymic reactivation (aged thymus) have higher levels 15 of HSV infection. It is well known that the mouse immune system is very similar to the human immune system, and results in mice can be projected to show human responses. This is reinforced by the data shown above of the effects of thymic reactivation in humans.

**[0123]** An additional step, supplying native hematopoietic cells to the patient during thymic reactivation, increases the immune capabilities of the patient's body. In particular, the immune system can be made to react specifically against various antigens by administering 20 genetically modified cells to a recipient. The genetically modified cells may be hematopoietic stem cells (HSC), epithelial stem cells, or hematopoietic progenitor cells. Preferably the genetically modified cells are CD34<sup>+</sup> HSC, lymphoid progenitor cells, or myeloid progenitor cells. Most preferably the genetically modified cells are CD34<sup>+</sup> HSC. The genetically modified cells are administered to the patient and migrate through the peripheral blood system 25 to the thymus. The uptake into the thymus of these hematopoietic precursor cells is substantially increased in the absence of sex steroids. These cells become integrated into the thymus and produce dendritic cells and T cells carrying the genetic modification from the altered cells. The results are a population of T cells with the desired genetic change that circulate in the peripheral blood of the recipient, and the accompanying increase in the 30 population of cells, tissues and organs caused by reactivation of the patient's thymus, which are capable of rapid, specific responses to antigen.

EFFECTS ON THE BONE MARROW AND HSC

**[0124]** The present disclosure provides methods for increasing the production of bone marrow in a patient, including increasing production of HSC. This is useful in a number of applications. For example, one of the difficult side effects of chemotherapy, whether given for cancer or for another purpose, can be its negative impact on the patient's bone marrow.

5 Depending on the dose of chemotherapy, the bone marrow may be ablated, and production of blood cells may be impeded. Administration of a dose of LHRH analog according to this invention after chemotherapy treatment will help to reverse the damage done by the chemotherapy to the bone marrow and blood cells. Alternatively, administration of the LHRH analog prior to delivery of chemotherapy will increase the population of HSC and 10 other blood cells so that the impact of chemotherapy will be decreased.

**[0125]** In some chemotherapy regimens, such as high dose chemotherapy to treat any of the blood cancers, ablation of the bone marrow is a desired effect. The methods of this invention may be used immediately after ablation occurs to stimulate the bone marrow and increase the production of HSC and their progeny blood cells, so as to decrease the patient's 15 recovery time. Following administration of the chemotherapy, usually allowing one or more days for the chemotherapy to clear from the patient's body, a dose of LHRH analog according to the methods described herein is administered to the patient. This can be in conjunction with the administration of autologous or heterologous bone marrow or hematopoietic stem or progenitor cells, as well as other factors such as Stem Cell Factor.

20 **[0126]** Alternatively, a patient may have "tired" bone marrow and may not be producing sufficient numbers of HSC and other blood cells to produce normal quantities. This can be caused by a variety of conditions, including normal aging, prolonged infection, post-chemotherapy, post-radiation therapy, chronic disease states including cancer, genetic abnormalities, and immunosuppression induced in transplantation. Further, radiation, such as 25 whole-body radiation, can have a major impact on the bone marrow productivity. These conditions can also be either pre-treated to minimize the negative effects (such as for chemotherapy and/or radiationtherapy), or treated after occurrence to reverse the effects.

#### SMALL ANIMAL STUDIES

##### Materials and Methods

30 **Animals**

**[0127]** CBA/CAH and C57Bl6/J male mice were obtained from Central Animal Services, Monash University and were housed under conventional conditions. Ages ranged from 4-6 weeks to 26 months of age and are indicated where relevant.

Castration

5 **[0128]** Animals were anesthetized by intraperitoneal injection of 0.3 ml of 0.3 mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5 mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline. Surgical castration was performed by a scrotal incision, revealing the testes, which were tied with suture and then removed along with surrounding fatty tissue.

10 Bromodeoxyuridine (BrdU) incorporation

**[0129]** Mice received two intraperitoneal injections of BrdU (Sigma Chemical Co., St. Louis, MO) (100 mg/kg body weight in 100 $\mu$ l of PBS) at a 4 hour interval. Control mice received vehicle alone injections. One hour after the second injection, thymuses were dissected and either a cell suspension made for FACS analysis, or immediately embedded in 15 Tissue Tek (O.C.T. compound, Miles INC, Indiana), snap frozen in liquid nitrogen, and stored at -70°C until use.

Flow Cytometric analysis

20 **[0130]** Mice were killed by CO<sub>2</sub> asphyxiation and thymus, spleen and mesenteric lymph nodes were removed. Organs were pushed gently through a 200 $\mu$ m sieve in cold PBS/1% FCS/0.02% Azide, centrifuged (650g, 5 min, 4°C), and resuspended in either PBS/FCS/Az. Spleen cells were incubated in red cell lysis buffer (8.9g/liter ammonium chloride) for 10 min at 4°C, washed and resuspended in PBS/FCS/Az. Cell concentration and viability were determined in duplicate using a hemocytometer and ethidium bromide/acridine orange and viewed under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, 25 Germany).

30 **[0131]** For 3-color immunofluorescence thymocytes were routinely labeled with anti- $\alpha\beta$ TCR-FITC or anti- $\gamma\delta$  TCR-FITC, anti-CD4-PE and anti-CD8-APC (all obtained from Pharmingen, San Diego, CA) followed by flow cytometry analysis. Spleen and lymph node suspensions were labeled with either  $\alpha\beta$ TCR-FITC/CD4-PE/CD8-APC or B220-B (Sigma) with CD4-PE and CD8-APC. B220-B was revealed with streptavidin-Tri-color conjugate purchased from Caltag Laboratories, Inc., Burlingame, CA.

[0132] For BrdU detection, cells were surface labeled with CD4-PE and CD8-APC, followed by fixation and permeabilization as previously described (Carayon and Bord, 1989). Briefly, stained cells were fixed O/N at 4°C in 1% PFA/0.01% Tween-20. Washed cells were incubated in 500µl DNase (100 Kunitz units, Boehringer Mannheim, W. Germany) for 30 5 mins at 37°C in order to denature the DNA. Finally, cells were incubated with anti-BrdU-FITC (Becton-Dickinson).

[0133] For 4-color Immunofluorescence thymocytes were labeled for CD3, CD4, CD8, B220 and Mac-1, collectively detected by anti-rat Ig-Cy5 (Amersham, U.K.), and the negative cells (TN) gated for analysis. They were further stained for CD25-PE (Pharmingen) 10 and CD44-B (Pharmingen) followed by Streptavidin-Tri-colour (Caltag, CA) as previously described (Godfrey and Zlotnik, 1993). BrdU detection was then performed as described above.

[0134] Samples were analyzed on a FacsCalibur (Becton-Dickinson). Viable lymphocytes were gated according to 0° and 90° light scatter profiles and data was analyzed 15 using Cell quest software (Becton-Dickinson).

#### Immunohistology

[0135] Frozen thymus sections (4µm) were cut using a cryostat (Leica) and immediately fixed in 100% acetone.

[0136] For two-color immunofluorescence, sections were double-labeled with a panel 20 of monoclonal antibodies: MTS6, 10, 12, 15, 16, 20, 24, 32, 33, 35 and 44 (Godfrey *et al.*, 1990; Table 1) produced in this laboratory and the co-expression of epithelial cell determinants was assessed with a polyvalent rabbit anti-cytokeratin Ab (Dako, Carpinteria, CA). Bound mAb was revealed with FITC-conjugated sheep anti-rat Ig (Silenus Laboratories) and anti-cytokeratin was revealed with TRITC-conjugated goat anti-rabbit Ig 25 (Silenus Laboratories).

[0137] For BrdU detection, sections were stained with either anti-cytokeratin followed by anti-rabbit-TRITC or a specific mAb, which was then revealed with anti-rat Ig-Cy3 (Amersham). BrdU detection was then performed as previously described (Penit *et al.*, 1996). Briefly, sections were fixed in 70% Ethanol for 30 mins. Semi-dried sections were incubated 30 in 4M HCl, neutralized by washing in Borate Buffer (Sigma), followed by two washes in PBS. BrdU was detected using anti-BrdU-FITC (Becton-Dickinson).

[0138] For three-color immunofluorescence, sections were labeled for a specific MTS mAb together with anti-cytokeratin. BrdU detection was then performed as described above.

[0139] Sections were analyzed using a Leica fluorescent and Nikon confocal microscopes.

5 Migration studies

[0140] Animals were anesthetized by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline.

10 [0141] Details of the FITC labeling of thymocytes technique are similar to those described elsewhere (Scollay *et al.*, 1980; Berzins *et al.*, 1998). Briefly, thymic lobes were exposed and each lobe was injected with approximately 10 $\mu$ m of 350  $\mu$ g/ml FITC (in PBS). The wound was closed with a surgical staple, and the mouse was warmed until fully recovered from anaesthesia. Mice were killed by CO<sub>2</sub> asphyxiation approximately 24h after injection and lymphoid organs were removed for analysis.

15 [0142] After cell counts, samples were stained with anti-CD4-PE and anti-CD8-APC, then analyzed by flow cytometry. Migrant cells were identified as live-gated FITC<sup>+</sup> cells expressing either CD4 or CD8 (to omit autofluorescing cells and doublets). The percentages of FITC<sup>+</sup> CD4 and CD8 cells were added to provide the total migrant percentage for lymph nodes and spleen, respectively. Calculation of daily export rates was performed as described by Berzins *et al.* (1998).

20 [0143] Data analyzed using the unpaired student 't' test or nonparametrical Mann-Whitney test was used to determine the statistical significance between control and test results for experiments performed at least in triplicate. Experimental values significantly differing from control values are indicated as follows: \*p $\leq$  0.05, \*\*p $\leq$  0.01 and \*\*\*p $\leq$  0.001.

25 Results

The effect of age on thymocyte populations.

(i) Thymic weight and thymocyte number

30 [0144] With increasing age there is a highly significant (p $\leq$ 0.0001) decrease in both thymic weight (Figure 1A) and total thymocyte number (Figure 1B). Relative thymic weight (mg thymus/g body) in the young adult has a mean value of 3.34 which decreases to 0.66 at 18-24 months of age (adipose deposition limits accurate calculation). The decrease in thymic weight can be attributed to a decrease in total thymocyte numbers: the 1-2 month thymus

contains  $\sim 6.7 \times 10^7$  thymocytes, decreasing to  $\sim 4.5 \times 10^6$  cells by 24 months. By removing the effects of sex steroids on the thymus by castration, regeneration occurs and by 4 weeks post-castration, the thymus is equivalent to that of the young adult in both weight and cellularity (Figure 1A and 1B). Interestingly, there is a significant ( $p \leq 0.001$ ) increase in 5 thymocyte numbers at 2 weeks post-castration ( $\sim 1.2 \times 10^8$ ), which is restored to normal young levels by 4 weeks post-castration (Figure 1B).

**[0145]** The decrease in T cell numbers produced by the thymus is not reflected in the periphery, with spleen cell numbers remaining constant with age (Figure 2A). Homeostatic mechanisms in the periphery were evident since the B cell to T cell ratio in spleen and lymph 10 nodes was not affected with age and the subsequent decrease in T cell numbers reaching the periphery (Figure 2B). However, the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cell significantly decreased ( $p \leq 0.001$ ) with age from 2:1 at 2 months of age, to a ratio of 1:1 at 2 years of age (Figure 2C). Following castration and the subsequent rise in T cell numbers reaching the periphery, no 15 change in peripheral T cell numbers was observed: splenic T cell numbers and the ratio of B:T cells in both spleen and lymph nodes was not altered following castration (Figure 2A and B). The decreased CD4:CD8 ratio in the periphery with age was still evident at 2 weeks post-castration but was completely reversed by 4 weeks post-castration (Figure 2C).

(ii)  $\alpha\beta$ TCR,  $\gamma\delta$ TCR, CD4 and CD8 expression

**[0146]** To determine if the decrease in thymocyte numbers seen with age was the 20 result of the depletion of specific cell populations, thymocytes were labeled with defining markers in order to analyze the separate subpopulations. In addition, this allowed analysis of the kinetics of thymus repopulation post-castration. The proportion of the main thymocyte subpopulations was compared with those of the normal young thymus (Figure 3) and found to remain uniform with age. In addition, further subdivision of thymocytes by the expression of 25  $\alpha\beta$ TCR and  $\gamma\delta$ TCR revealed no change in the proportions of these populations with age (data not shown). At 2 and 4 weeks post-castration, thymocyte subpopulations remained in the same proportions and, since thymocyte numbers increase by up to 100-fold post-castration, this indicates a synchronous expansion of all thymocyte subsets rather than a developmental progression of expansion.

**[0147]** The decrease in cell numbers seen in the thymus of aged animals thus appears 30 to be the result of a balanced reduction in all cell phenotypes, with no significant changes in T

cell populations being detected. Thymus regeneration occurs in a synchronous fashion, replenishing all T cell subpopulations simultaneously rather than sequentially.

#### Proliferation of thymocytes

**[0148]** As shown in Figure 4, 15-20% of thymocytes are proliferating at 4-6 weeks of age. The majority (~80%) of these are DP with the TN subset making up the second largest population at ~6% (Figure 5A). Accordingly, most division is seen in the subcapsule and cortex by immunohistology (data not shown). Some division is seen in the medullary regions with FACS analysis revealing a proportion of SP cells (9% of CD4 T cells and 25% of CD8 T cells) dividing (Figure 5B).

**[0149]** Although cell numbers are significantly decreased in the aged thymus, proliferation of thymocytes remains constant, decreasing to 12-15% at 2 years (Figure 4), with the phenotype of the proliferating population resembling the 2 month thymus (Figure 5A). Immunohistology revealed the division at 1 year of age to reflect that seen in the young adult; however, at 2 years, proliferation is mainly seen in the outer cortex and surrounding the vasculature (data not shown). At 2 weeks post-castration, although thymocyte numbers significantly increase, there is no change in the proportion of thymocytes that are proliferating, again indicating a synchronous expansion of cells (Figure 4). Immunohistology revealed the localization of thymocyte proliferation and the extent of dividing cells to resemble the situation in the 2-month-old thymus by 2 weeks post-castration (data not shown). When analyzing the proportion of each subpopulation which represent the proliferating population, there was a significant ( $p<0.001$ ) increase in the percentage of CD8 T cells which are within the proliferating population (1% at 2 months and 2 years of age, increasing to ~6% at 2 weeks post-castration) (Figure 5A).

**[0150]** Figure 5B illustrates the extent of proliferation within each subset in young, old and castrated mice. There is a significant ( $p\leq 0.001$ ) decay in proliferation within the DN subset (35% at 2 months to 4% by 2 years). Proliferation of CD8+ T cells was also significantly ( $p\leq 0.001$ ) decreased, reflecting the findings by immunohistology (data not shown) where no division is evident in the medulla of the aged thymus. The decrease in DN proliferation is not returned to normal young levels by 4 weeks post-castration. However, proliferation within the CD8+ T cell subset is significantly ( $p\leq 0.001$ ) increased at 2 weeks post-castration and is returning to normal young levels at 4 weeks post-castration.

[0151] The decrease in proliferation within the DN subset was analyzed further using the markers CD44 and CD25. The DN subpopulation, in addition to the thymocyte precursors, contains  $\alpha\beta$ TCR+CD4-CD8- thymocytes, which are thought to have downregulated both co-receptors at the transition to SP cells (Godfrey & Zlotnik, 1993). By 5 gating on these mature cells, it was possible to analyze the true TN compartment (CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>) and these showed no difference in their proliferation rates with age or following castration (Figure 5C). However, analysis of the subpopulations expressing CD44 and CD25, showed a significant ( $p<0.001$ ) decrease in proliferation of the TN1 subset (CD44<sup>+</sup>CD25<sup>-</sup>), from 20% in the normal young to around 6% at 18 months of age (Figure 5D) which was 10 restored by 4 weeks post-castration. The decrease in the proliferation of the TN1 subset, was compensated for by a significant ( $p\leq 0.001$ ) increase in proliferation of the TN2 subpopulation (CD44<sup>+</sup>CD25<sup>+</sup>) which returned to normal young levels by 2 weeks post-castration (Figure 5D).

The effect of age on the thymic microenvironment.

[0152] The changes in the thymic microenvironment with age were examined by immunofluorescence using an extensive panel of MAbs from the MTS series, double-labeled with a polyclonal anti-cytokeratin Ab.

[0153] The antigens recognized by these MAbs can be subdivided into three groups: thymic epithelial subsets, vascular-associated antigens and those present on both stromal cells 20 and thymocytes.

(i) Epithelial cell antigens.

[0154] Anti-keratin staining (pan-epithelium) of 2 year old mouse thymus, revealed a loss of general thymus architecture with a severe epithelial cell disorganization and absence of a distinct cortico-medullary junction. Further analysis using the MAbs, MTS 10 (medulla) 25 and MTS44 (cortex), showed a distinct reduction in cortex size with age, with a less substantial decrease in medullary epithelium (data not shown). Epithelial cell free regions, or keratin negative areas (KNA's, van Ewijk *et al.*, 1980; Godfrey *et al.*, 1990; Bruijntjes *et al.*, 1993.) were more apparent and increased in size in the aged thymus, as evident with anti-cytokeratin labeling. There is also the appearance of thymic epithelial "cyst-like" structures 30 in the aged thymus particularly noticeable in medullary regions (data not shown). Adipose deposition, severe decrease in thymic size and the decline in integrity of the cortico-medullary junction are shown conclusively with the anti-cytokeratin staining (data not shown). The

thymus is beginning to regenerate by 2 weeks post-castration. This is evident in the size of the thymic lobes, the increase in cortical epithelium as revealed by MTS 44, and the localization of medullary epithelium. The medullary epithelium is detected by MTS 10 and at 2 weeks, there are still subpockets of epithelium stained by MTS 10 scattered throughout the cortex. By 4 weeks post-castration, there is a distinct medulla and cortex and discernible cortico-medullary junction (data not shown).

5 [0155] The markers MTS 20 and 24 are presumed to detect primordial epithelial cells (Godfrey, *et al.*, 1990) and further illustrate the degeneration of the aged thymus. These are present in abundance at E14, detect isolated medullary epithelial cell clusters at 4-6 weeks but 10 are again increased in intensity in the aged thymus (data not shown). Following castration, all these antigens are expressed at a level equivalent to that of the young adult thymus (data not shown) with MTS 20 and MTS 24 reverting to discrete subpockets of epithelium located at the cortico-medullary junction.

(ii) Vascular-associated antigens.

15 [0156] The blood-thymus barrier is thought to be responsible for the immigration of T cell precursors to the thymus and the emigration of mature T cells from the thymus to the periphery.

20 [0157] The MAb MTS 15 is specific for the endothelium of thymic blood vessels, demonstrating a granular, diffuse staining pattern (Godfrey, *et al.*, 1990). In the aged thymus, MTS 15 expression is greatly increased, and reflects the increased frequency and size of blood vessels and perivascular spaces (data not shown).

25 [0158] The thymic extracellular matrix, containing important structural and cellular adhesion molecules such as collagen, laminin and fibrinogen, is detected by the mAb MTS 16. Scattered throughout the normal young thymus, the nature of MTS 16 expression becomes more widespread and interconnected in the aged thymus. Expression of MTS 16 is increased further at 2 weeks post-castration while 4 weeks post-castration, this expression is representative of the situation in the 2 month thymus (data not shown).

(iii) Shared antigens

30 [0159] MHC II expression in the normal young thymus, detected by the MAb MTS 6, is strongly positive (granular) on the cortical epithelium (Godfrey *et al.*, 1990) with weaker staining of the medullary epithelium. The aged thymus shows a decrease in MHC II expression with expression substantially increased at 2 weeks post-castration. By 4 weeks

post-castration, expression is again reduced and appears similar to the 2 month old thymus (data not shown).

#### Thymocyte emigration

[0160] Approximately 1% of T cells migrate from the thymus daily in the young mouse (Scollay *et al.*, 1980). We found migration was occurring at a proportional rate equivalent to the normal young mouse at 14 months and even 2 years of age (Figure 5) although significantly ( $p \leq 0.0001$ ) reduced in number. There was an increase in the CD4:CD8 ratio of the recent thymic emigrants from ~3:1 at 2 months to ~7:1 at 26 months. By 1 week post-castration, cell number migrating to the periphery has substantially increased with the overall rate of migration remaining constant at 1-1.5%.

#### EXAMPLES

[0161] The following Examples provide specific examples of methods of the invention, and are not to be construed as limiting the invention to their content. For convenience these examples describe delivery of an LHRH agonist to block sex steroid mediated signaling to the thymus as well as the resultant prevention of HSV infection.

##### EXAMPLE 1

###### SEX STEROID ABLATION THERAPY

[0162] In the event that an unmatched donor is involved, the patient is given immunosuppressive therapy, such as cyclosporin therapy, to prevent rejection of donor cells.

[0163] The patient was given sex steroid ablation therapy in the form of delivery of an LHRH agonist. This was given in the form of either Leucrin (depot injection; 22.5mg) or Zoladex (implant; 10.8 mg), either one as a single dose effective for 3 months. This was effective in reducing sex steroid levels sufficiently to reactivate the thymus. In some cases it is also necessary to deliver a suppresser of adrenal gland production of sex steroids, such as Cosudex (5mg/day) as one tablet per day for the duration of the sex steroid ablation therapy. Adrenal gland production of sex steroids makes up around 10-15% of a human's steroids.

[0164] Reduction of sex steroids in the blood to minimal values took about 1-3 weeks; concordant with this was the reactivation of the thymus. In some cases it is necessary to extend the treatment to a second 3 month injection/implant.

##### EXAMPLE 2

###### ALTERNATIVE DELIVERY METHOD

**[0165]** In place of the 3 month depot or implant administration of the LHRH agonist, alternative methods can be used. In one example the patient's skin may be irradiated by a laser such as an Er:YAG laser, to ablate or alter the skin so as to reduce the impeding effect of the stratum corneum.

5 **[0166]** A. Laser Ablation or Alteration: An infrared laser radiation pulse was formed using a solid state, pulsed, Er:YAG laser consisting of two flat resonator mirrors, an Er:YAG crystal as an active medium, a power supply, and a means of focusing the laser beam. The wavelength of the laser beam was 2.94 microns. Single pulses were used.

10 **[0167]** The operating parameters were as follows: The energy per pulse was 40, 80 or 120 mJ, with the size of the beam at the focal point being 2 mm, creating an energy fluence of 1.27, 2.55 or 3.82 J/cm<sup>2</sup>. The pulse temporal width was 300  $\mu$ s, creating an energy fluence rate of 0.42, 0.85 or  $1.27 \times 10^4$  W/cm<sup>2</sup>.

15 **[0168]** Subsequently, an amount of LHRH agonist is applied to the skin and spread over the irradiation site. The LHRH agonist may be in the form of an ointment so that it remains on the site of irradiation. Optionally, an occlusive patch is placed over the agonist in order to keep it in place over the irradiation site.

20 **[0169]** Optionally a beam splitter is employed to split the laser beam and create multiple sites of ablation or alteration. This provides a faster flow of LHRH agonist through the skin into the blood stream. The number of sites can be predetermined to allow for maintenance of the agonist within the patient's system for the requisite approximately 30 days.

25 **[0170]** B. Pressure Wave: A dose of LHRH agonist is placed on the skin in a suitable container, such as a plastic flexible washer (about 1 inch in diameter and about 1/16 inch thick), at the site where the pressure wave is to be created. The site is then covered with target material such as a black polystyrene sheet about 1 mm thick. A Q-switched solid state ruby laser (20 ns pulse duration, capable of generating up to 2 joules per pulse) is used to generate the laser beam, which hits the target material and generates a single impulse transient. The black polystyrene target completely absorbs the laser radiation so that the skin is exposed only to the impulse transient, and not laser radiation. No pain is produced from this procedure. The procedure can be repeated daily, or as often as required, to maintain the circulating blood levels of the agonist.

EXAMPLE 3

## ADMINISTRATION OF HSC TO ENHANCE THYMIC REACTIVATION

[0171] HSC are collected from the patient, or preferably from a donor. The cells are optionally increased in number by ex vivo culturing in the presence of Stem Cell Factor (SCF). At approximately 1-3 weeks post LHRH agonist delivery, just before or at the time 5 the thymus begins to reactivate, the patient is injected with the HSC, optimally at a dose of about  $2-4 \times 10^6$  cells/kg. Optionally G-CSF may also be injected into the patient to assist in expansion of the HSC. The reactivated thymus takes up the HSC and converts donor HSC into donor-type T cells and dendritic cells, and patient HSC into patient-type T cells and dendritic cells. By inducing deletion by cell death, or by inducing tolerance through 10 immunoregulatory cells, the donor dendritic cells will tolerize any T cells that are potentially reactive with recipient.

## EXAMPLE 4

## GENETIC MODIFICATION OF HSC TO PREVENT HIV INFECTION

[0172] Where practical, the level of HSC in the blood of the patient or a donor is 15 enhanced by injecting granulocyte-colony stimulating factor (G-CSF) at  $10\mu\text{g}/\text{kg}$  for 2-5 days prior to cell collection. CD34<sup>+</sup> donor cells are purified from the donor or patient blood or bone marrow, preferably using a flow cytometer or immunomagnetic beading. HSC are identified by flow cytometry as being CD34<sup>+</sup>. Optionally these HSC are expanded ex vivo with SCF.

[0173] A retroviral vector is constructed to contain the trans-dominant mutant form of 20 HIV-1 *rev* gene, RevM10, which has been shown to inhibit HIV replication (Bonyhadi *et al.* 1997). Amphotropic vector-containing supernatants are generated by infection with filtered supernatants from ecotropic producer cells that were transfected with the vector. The collected CD34<sup>+</sup> cells are prestimulated for 24 hours in LCTM media supplemented with IL- 25 3, IL-6 and SCF (10ng/ml each) to induce entry of the cells into the cell cycle. Supernatants containing the vectors are then repeatedly added to the cells for 2-3 days to allow transduction of the vectors into the cells.

[0174] At approximately 1-3 weeks post LHRH agonist delivery, just before or at the 30 time the thymus begins to reactivate, the patient is injected with the genetically modified HSC, optimally at a dose of about  $2-4 \times 10^6$  cells/kg. Optionally G-CSF may also be injected into the recipient to assist in expansion of the HSC. The reactivated thymus takes up the genetically modified HSC and converts them into donor-type T cells and dendritic cells, while

converting the recipient's HSC into recipient-type T cells and dendritic cells. By inducing deletion by cell death, or by inducing tolerance through immunoregulatory cells, the donor dendritic cells will tolerize any T cells that are potentially reactive with recipient.

EXAMPLE 5

5 TERMINATION OF IMMUNOSUPPRESSION WHEN DONOR CELLS ARE USED

[0175] When the thymic chimera is established and the new cohort of mature T cells have begun exiting the thymus, blood is taken from the patient and the T cells examined in vitro for their lack of responsiveness to donor cells in a standard mixed lymphocyte reaction. If there is no response, the immunosuppressive therapy is gradually reduced to allow defense 10 against infection. If there is no sign of rejection, as indicated in part by the presence of activated T cells in the blood, the immunosuppressive therapy is eventually stopped completely. Because the HSC have a strong self-renewal capacity, the hematopoietic chimera so formed will be stable theoretically for the life of the patient (as for normal, non-tolerized and non-grafted people).

15 EXAMPLE 6

USE OF LHRH AGONIST TO REACTIVATE THE THYMUS IN HUMANS

[0176] In order to show that a human thymus can be reactivated by the methods of this invention, these methods were used on patients who had been treated with chemotherapy for prostate cancer. Prostate cancer patients were evaluated before and 4 months after sex steroid 20 ablation therapy. The results are summarized in Figs 23 – 27. Collectively the data demonstrate qualitative and quantitative improvement of the status of T cells in many patients.

[0177] The effect of LHRH therapy on total numbers of lymphocytes and T cells subsets thereof:

25 [0178] The phenotypic composition of peripheral blood lymphocytes was analyzed in patients (all >60 years) undergoing LHRH agonist treatment for prostate cancer (Fig 23). Patient samples were analyzed before treatment and 4 months after beginning LHRH agonist treatment. Total lymphocyte cell numbers per ml of blood were at the lower end of control values before treatment in all patients. Following treatment, 6/9 patients showed substantial 30 increases in total lymphocyte counts (in some cases a doubling of total cells was observed). Correlating with this was an increase in total T cell numbers in 6/9 patients. Within the CD4<sup>+</sup> subset, this increase was even more pronounced with 8/9 patients demonstrating increased

levels of CD4<sup>+</sup> T cells. A less distinctive trend was seen within the CD8+ subset with 4/9 patients showing increased levels albeit generally to a smaller extent than CD4<sup>+</sup> T cells.

[0179] The Effect Of LHRH Therapy On The Proportion Of T Cells Subsets:

[0180] Analysis of patient blood before and after LHRH agonist treatment

5 demonstrated no substantial changes in the overall proportion of T cells, CD4<sup>+</sup> or CD8<sup>+</sup> T cells and a variable change in the CD4<sup>+</sup>:CD8<sup>+</sup> ratio following treatment (Fig 24). This indicates that there was little effect of treatment on the homeostatic maintenance of T cell subsets despite the substantial increase in overall T cell numbers following treatment. All values were comparative to control values.

10 [0181] The Effect Of LHRH Therapy On The Proportion Of B Cells And Myeloid Cells:

[0182] Analysis of the proportions of B cells and myeloid cells (NK, NKT and macrophages) within the peripheral blood of patients undergoing LHRH agonist treatment demonstrated a varying degree of change within subsets (Fig 25). While NK, NKT and 15 macrophage proportions remained relatively constant following treatment, the proportion of B cells was decreased in 4/9 patients.

[0183] The Effect Of LHRH Agonist Therapy On The Total Number Of B Cells And Myeloid Cells:

[0184] Analysis of the total cell numbers of B and myeloid cells within the peripheral 20 blood post-treatment showed clearly increased levels of NK (5/9 patients), NKT (4/9 patients) and macrophage (3/9 patients) cell numbers post-treatment (Fig 26). B cell numbers showed no distinct trend with 2/9 patients showing increased levels; 4/9 patients showing no change and 3/9 patients showing decreased levels.

[0185] The Effect Of LHRH Therapy On The Level Of Naïve Cells Relative To 25 Memory Cells:

[0186] The major changes seen post-LHRH agonist treatment were within the T cell population of the peripheral blood. In particular there was a selective increase in the proportion of naïve (CD45RA<sup>+</sup>) CD4+ cells, with the ratio of naïve (CD45RA<sup>+</sup>) to memory (CD45RO<sup>+</sup>) in the CD4<sup>+</sup> T cell subset increasing in 6/9 patients (Fig 27).

30 Conclusion

[0187] Thus it can be concluded that LHRH agonist treatment of an animal such as a human having an atrophied thymus can induce reactivation of the thymus. A general

improvement has been shown in the status of blood T lymphocytes in these prostate cancer patients who have received sex-steroid ablation therapy. While it is very difficult to precisely determine whether such cells are only derived from the thymus, this would be very much the logical conclusion as no other source of mainstream (CD8  $\alpha\beta$  chain) T cells has been 5 described. Gastrointestinal tract T cells are predominantly TCR  $\gamma\delta$  or CD8  $\alpha\alpha$  chain.

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## CLAIMS:

1. A method for prevention of infection of a patient by an infecting agent comprising reactivating the patient's thymus.
2. The method of claim 1 wherein the patient's thymus has been at least in part deactivated.
3. The method of claim 2 wherein the patient is post-pubertal.
4. The method of claim 2 wherein the patient has or had a disease or treatment of a disease that at least in part deactivated the patient's thymus.
5. The method of claim 1 wherein the reactivation is induced prior to or right after the patient is initially exposed to the infecting agent.
6. The method of claim 1 wherein reactivating the patient's thymus is accomplished through disruption of sex steroid mediated signaling to the thymus.
7. The method of claim 6 wherein the method of disrupting the sex steroid mediated signaling to the thymus is through administration of one or more pharmaceuticals that lower the concentration of sex steroids in a patient.
8. The method of claim 7 wherein the pharmaceuticals are selected from the group consisting of LHRH analogs, anti-LHRH vaccines, and combinations thereof.
9. The method of claim 8 wherein the LHRH analog is an LHRH agonist or an LHRH antagonist.
10. The method of claim 9 wherein the LHRH agonist is selected from the group consisting of Buserelin, Cystorelin, Decapeptyl, Deslorelin, Gonadorelin, Goserelin, Histrelin, Leuprolide, Leuprorelin, Lutrelin, Meterelin, Nafarelin and Triptorelin.
11. The method of claim 9 wherein the LHRH antagonist is selected from the group consisting of Eulexin and Abarelix.
12. The method of claim 6 wherein the method of disrupting the sex steroid mediated signaling to the thymus is through surgical castration of the patient.
13. The method of claim 7 having the further step of delivering at least one cytokine, at least one growth factor, or a combination of at least one cytokine and at least one growth factor to the patient.
14. The method of claim 13 wherein the cytokine is selected from the group consisting of Interleukin 2 (IL2), Interleukin 7 (IL7) and Interleukin 15 (IL15) and combinations thereof.

15. The method of claim 13 wherein the growth factor is selected from the group consisting of members of the epithelial growth factor family, members of the fibroblast growth factor family, Stem Cell Factor, granulocyte colony stimulating factor (GCSF),

5 keratinocyte growth factor (KGF), and combinations thereof.

16. The method of claim 13 wherein the cytokine and/or growth factor is delivered prior to delivery of the LHRH analog, the anti-LHRH vaccine, or the combination thereof.

17. The method of claim 13 wherein the cytokine and/or growth factor is delivered during or after delivery of the LHRH analog, the anti-LHRH vaccine, or the combination

10 thereof.

18. The method of claim 6 further comprising the step of delivering to the patient cells selected from the group consisting of HSC, myeloid progenitor cells, lymphoid progenitor cells and epithelial stem cells.

19. The method of claim 18 wherein the cells are delivered to the patient between about one and three weeks after disruption of sex steroid mediated signaling to the thymus.

20. The method of claim 18 wherein the cells are delivered at the time the thymus begins to be reactivated.

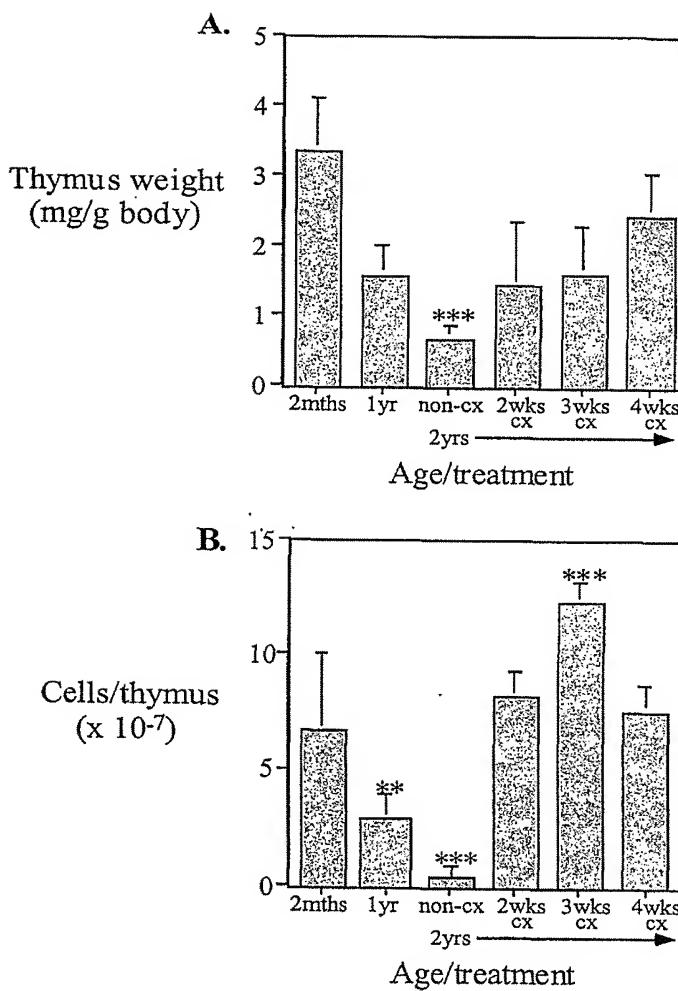
21. The method of claim 18 wherein the cells are genetically modified.

22. The method of claim 21 wherein the genetic modification creates resistance in the cells and their progeny to infection by an external agent.

23. The method of claim 22 wherein the external agent is a virus.

24. The method of claim 23 wherein the virus is selected from the group consisting of HIV, flu virus, hepatitis A virus, hepatitis B virus and hepatitis C virus.

25. A method for enhancing bone marrow productivity in a patient comprising the step of administering an LHRH analog to the patient.



**Figure 1**

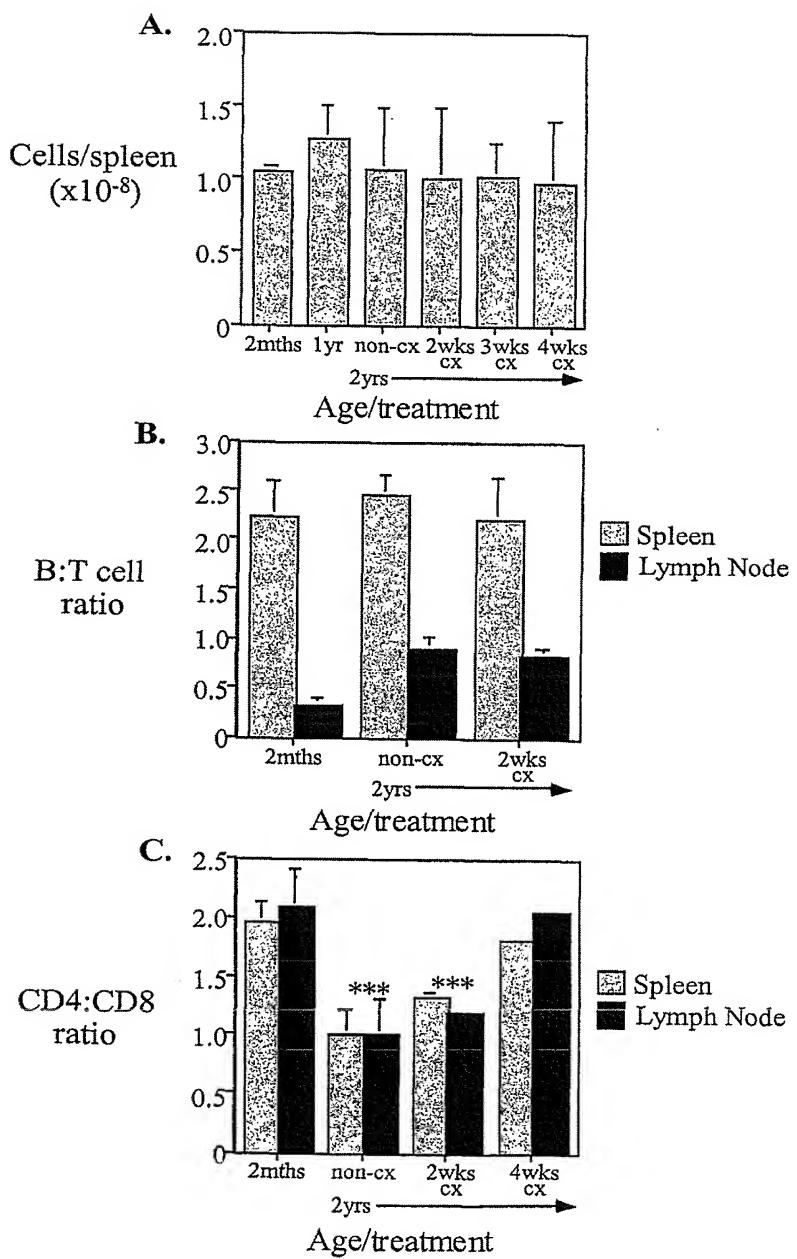
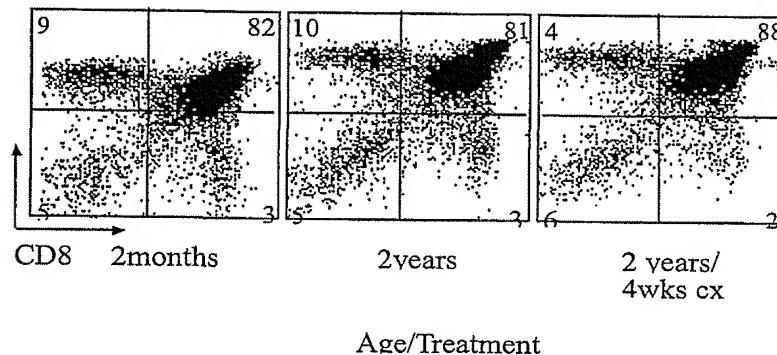
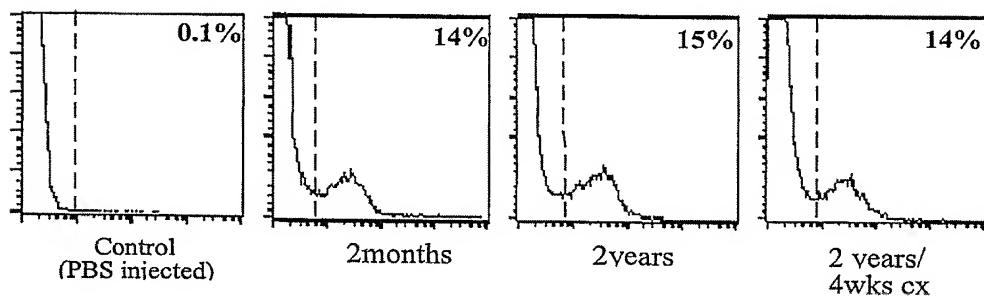


Figure 2

**Figure 3****Figure 4**

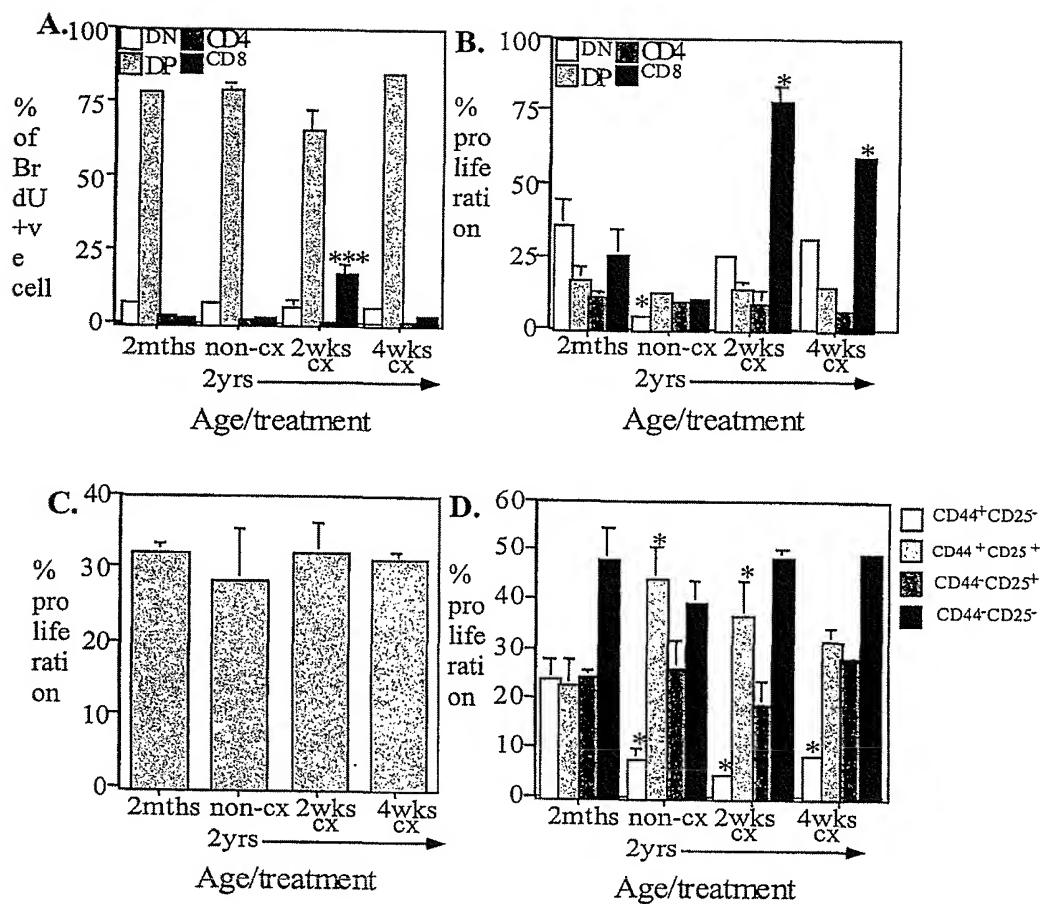


Figure 5

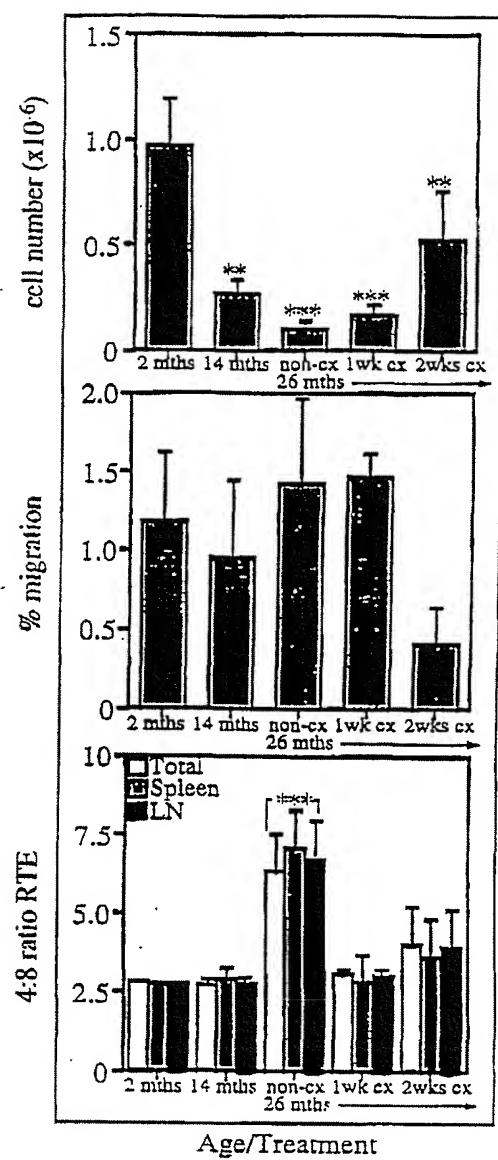
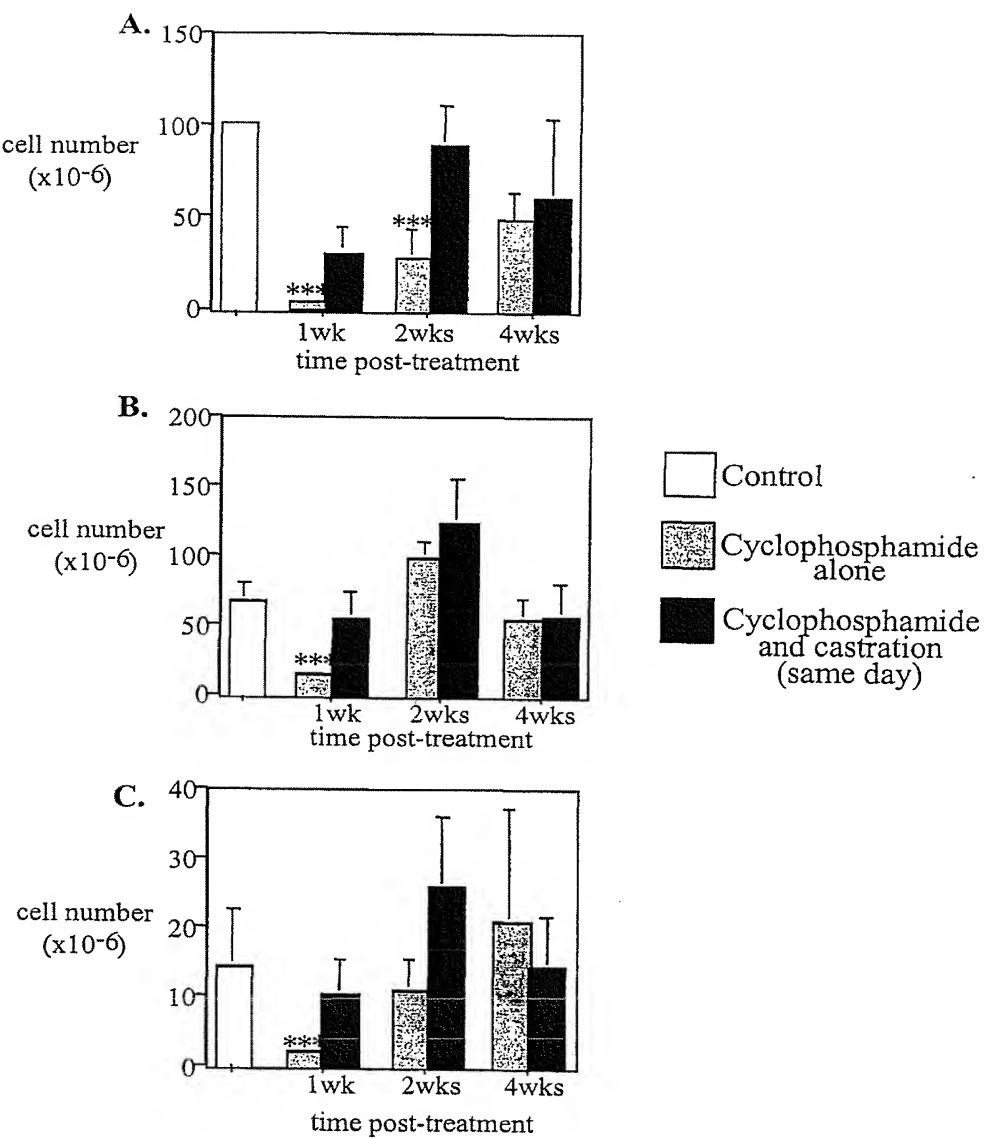
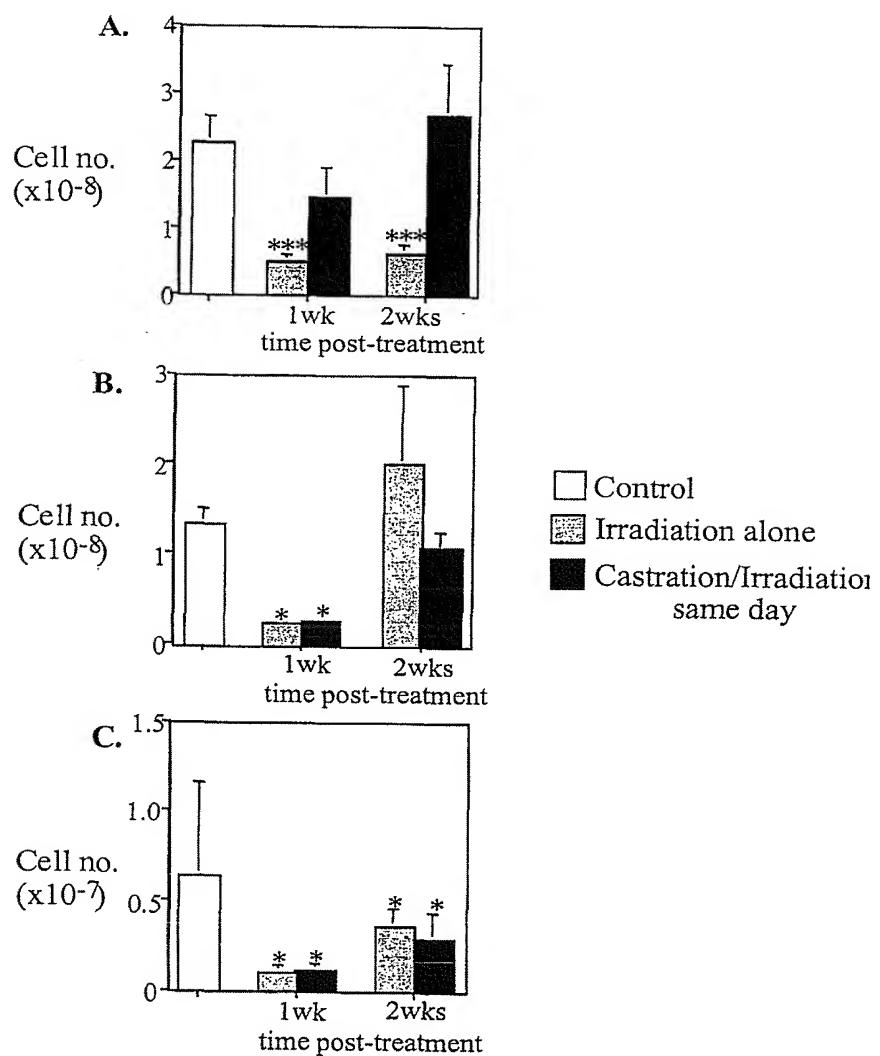
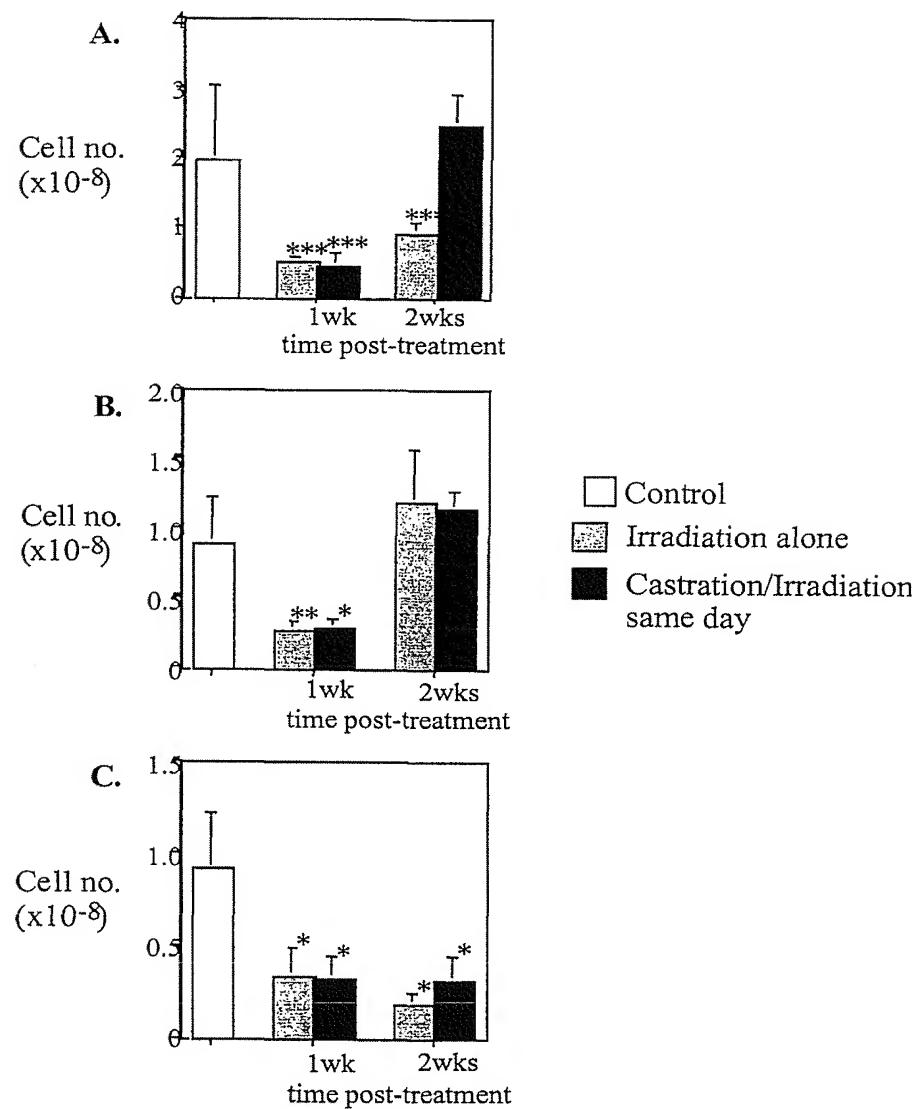


FIGURE 6

**Figure 7**



**Figure 8**

**Figure 9**

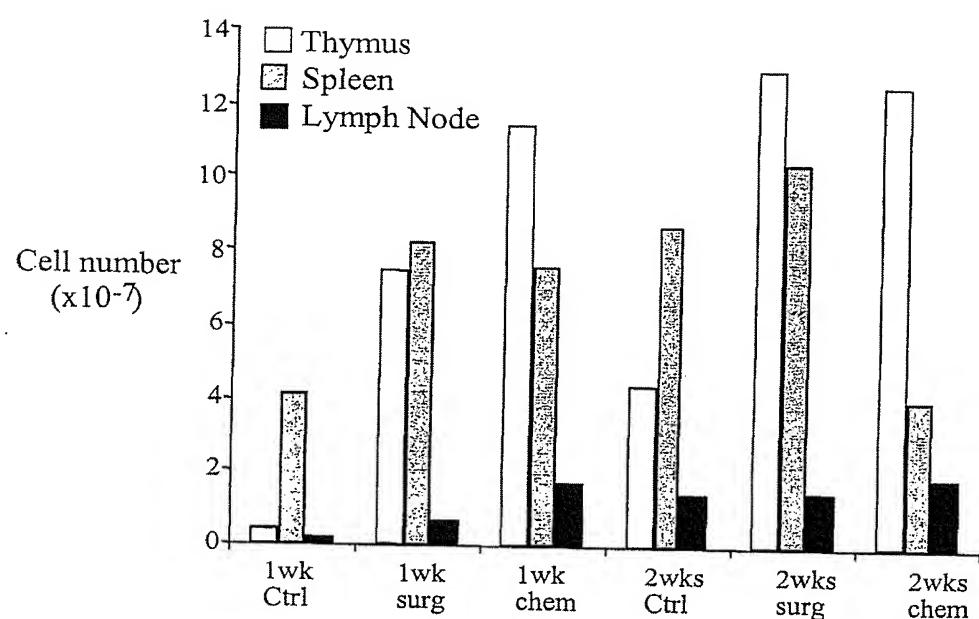
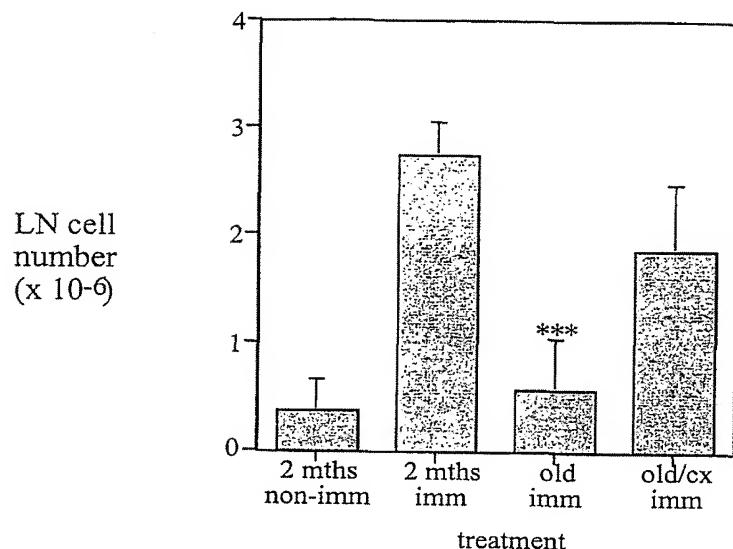


Figure 10

## LN size post-HSV inoculation



## Activated cell numbers

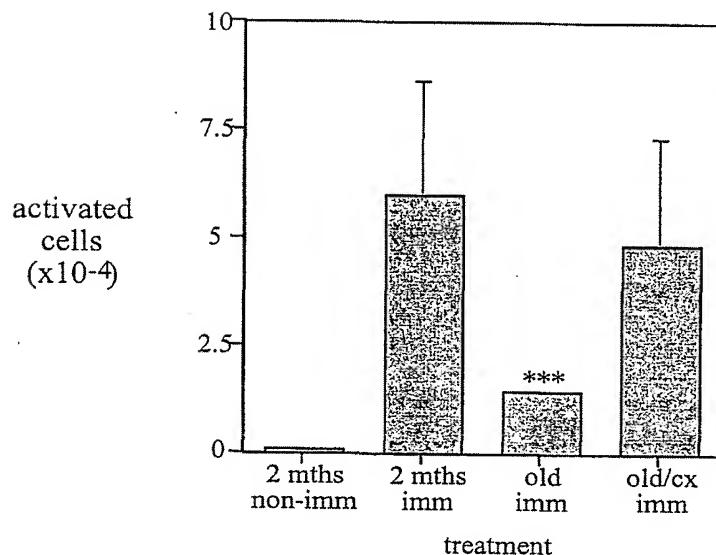
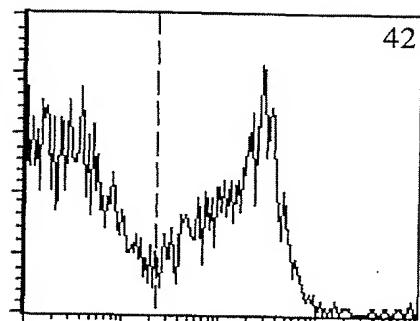
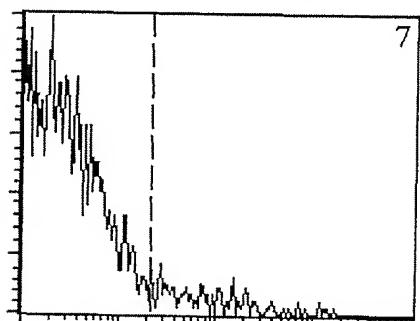


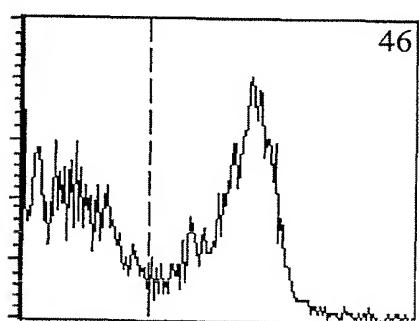
Figure 11



2 months

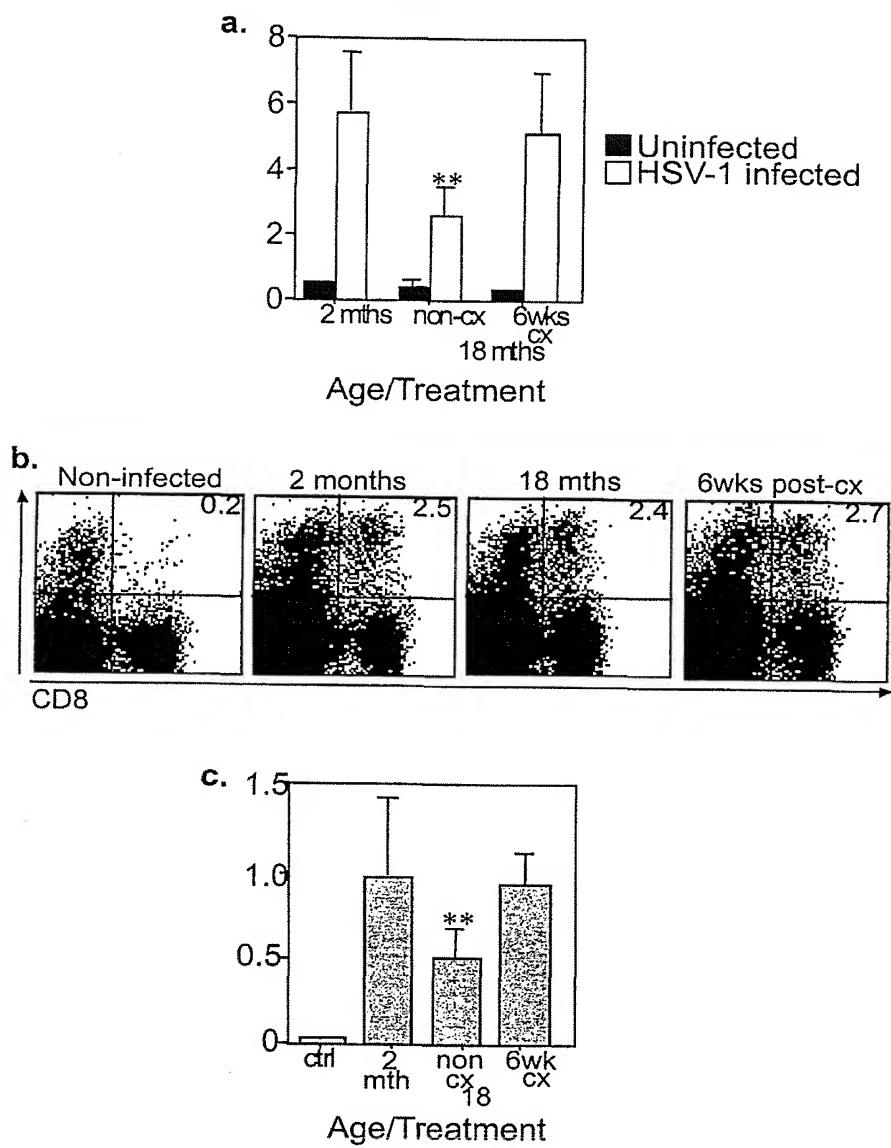


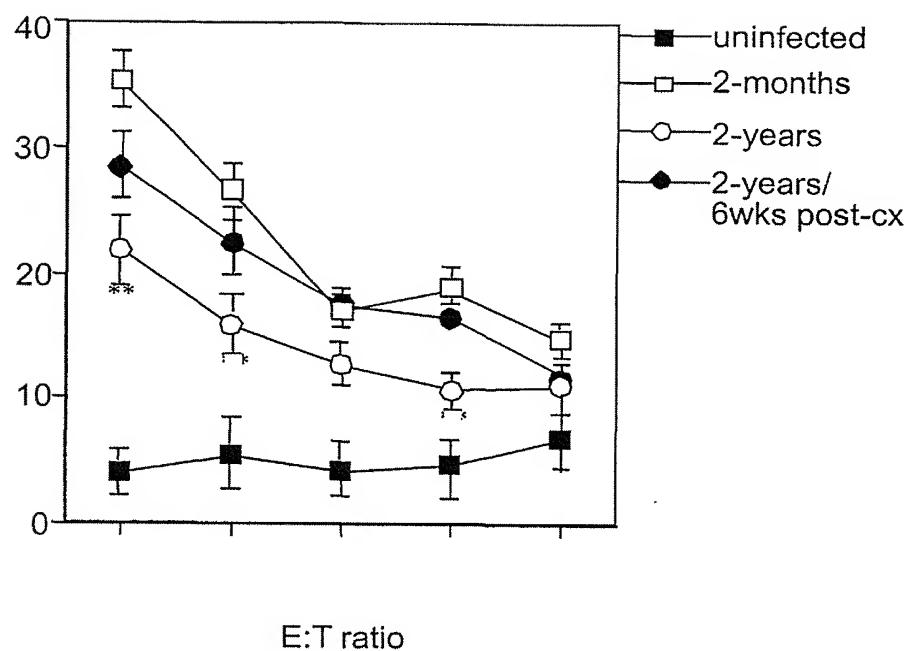
18 months

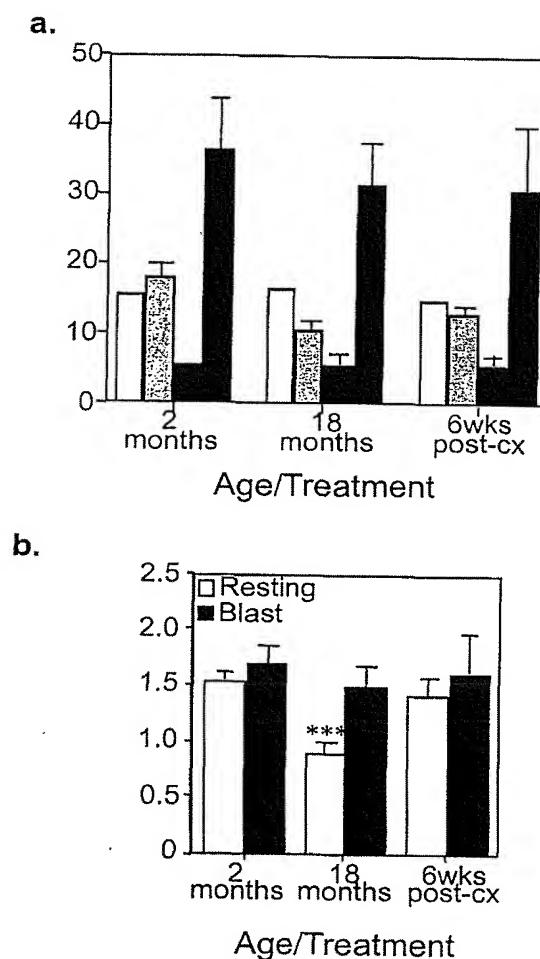


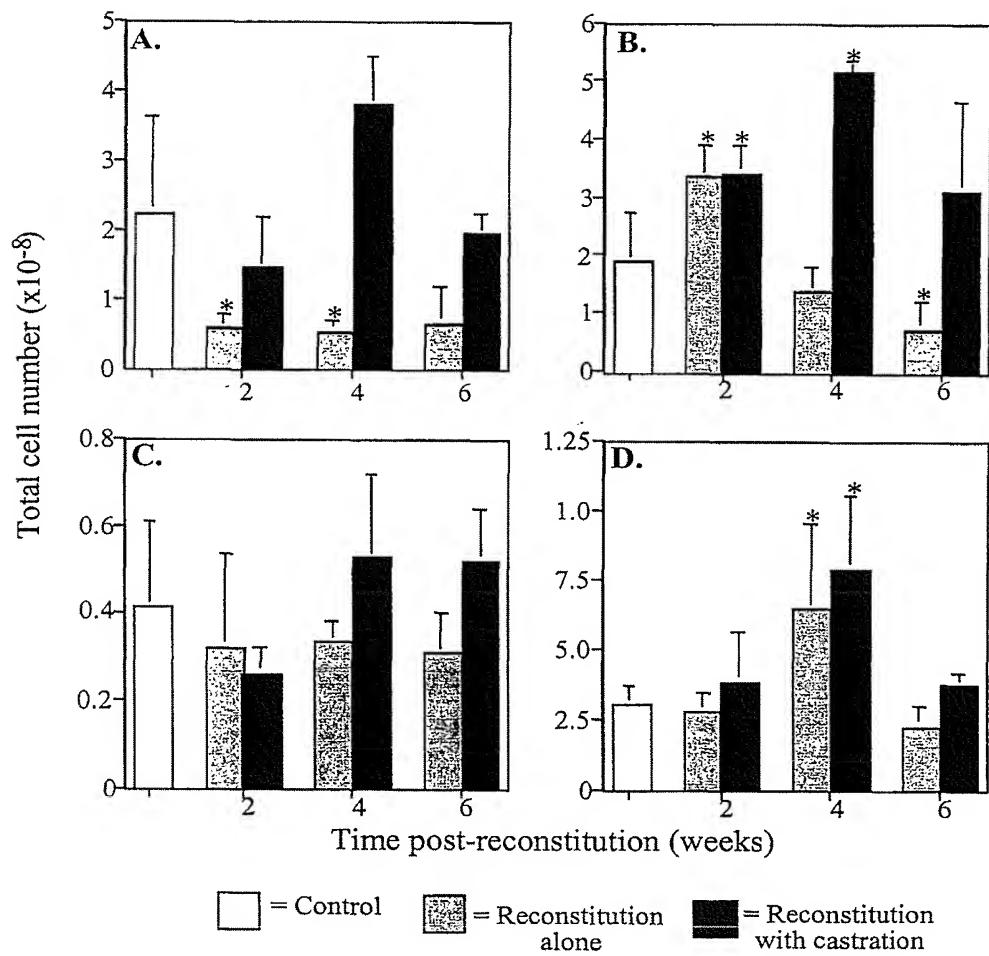
18 months  
Castrated

**Figure 12**

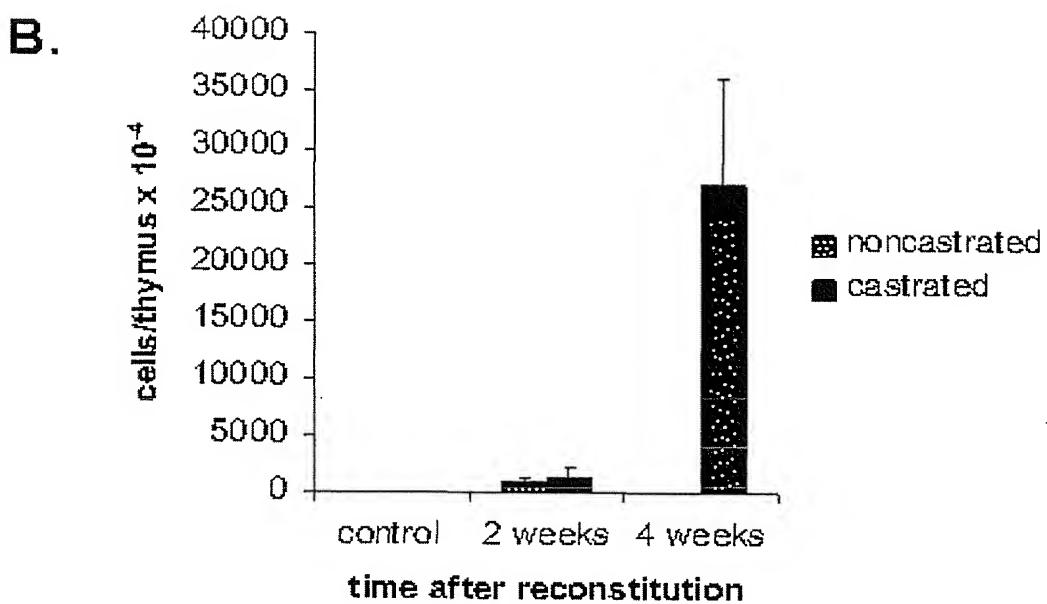
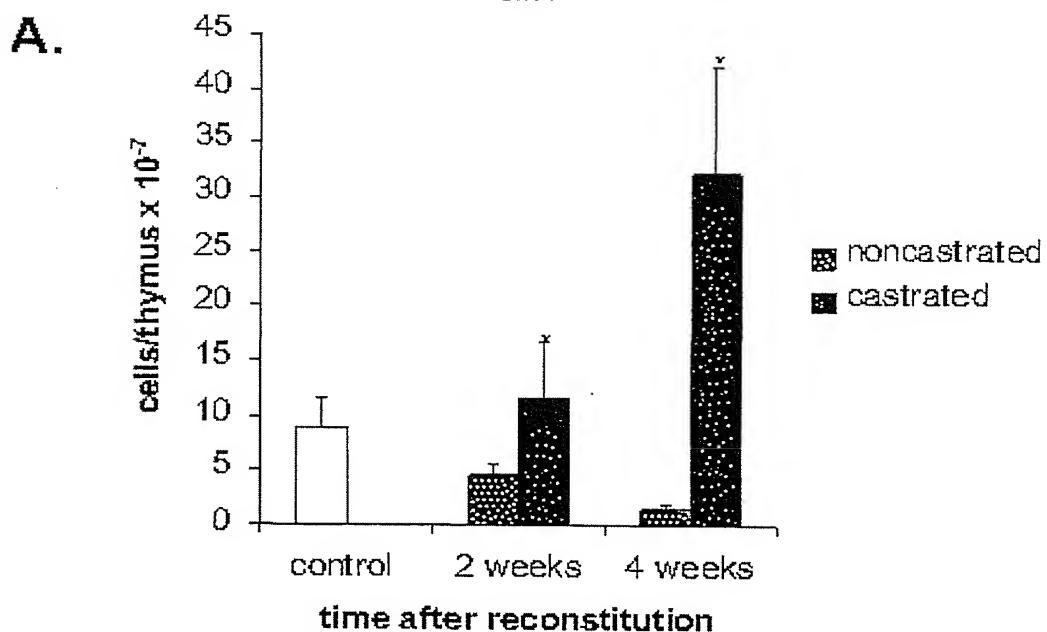
**FIGURE 13**

**FIGURE 14**

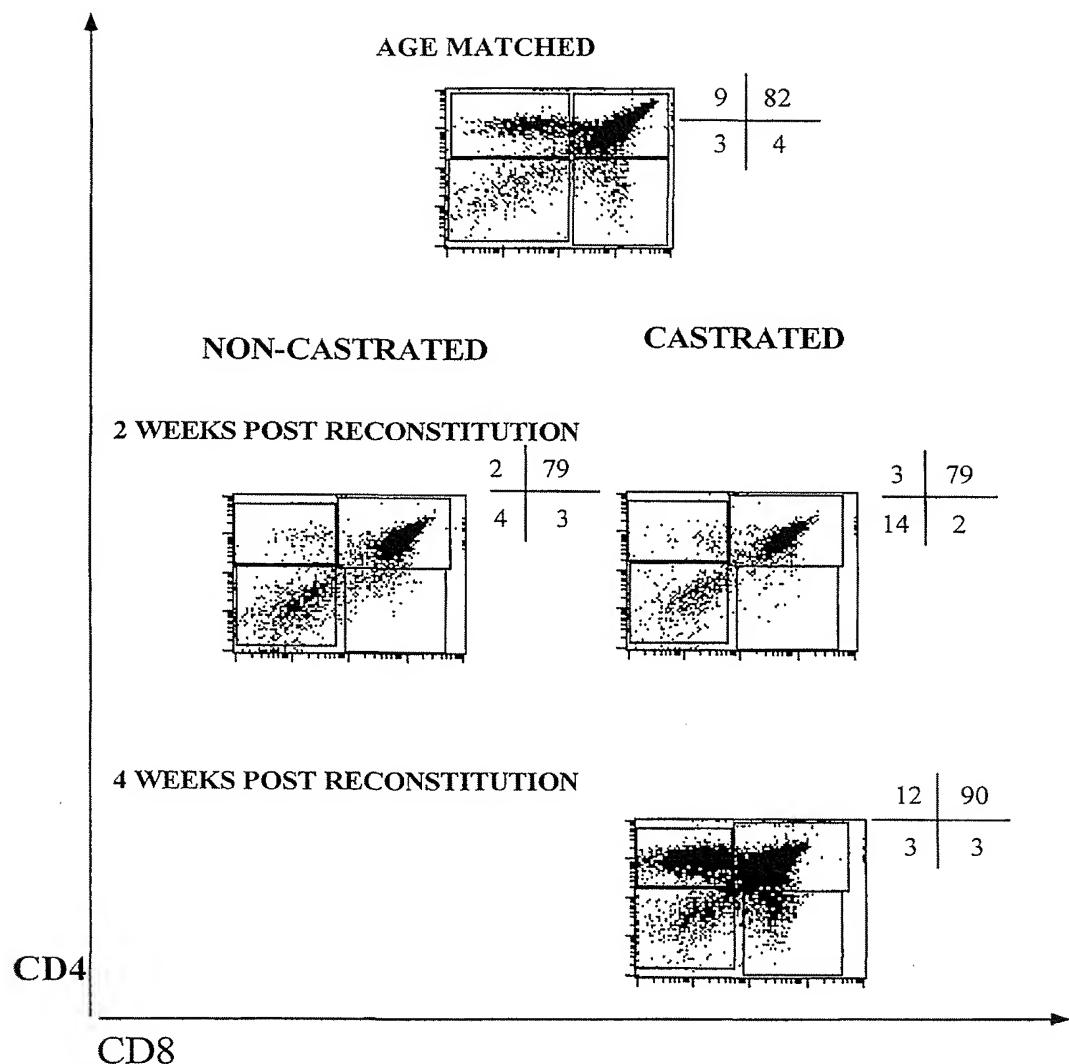
**FIGURE 15**

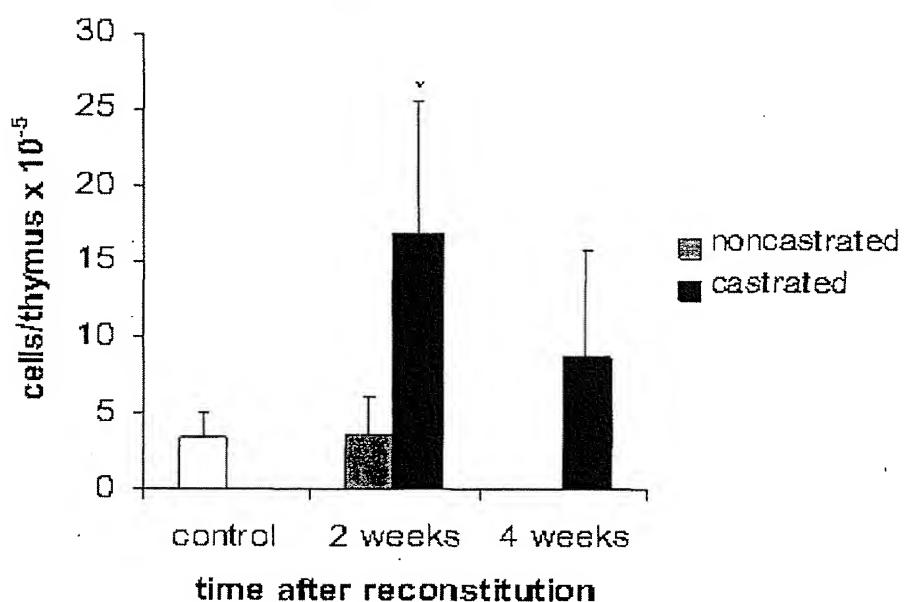
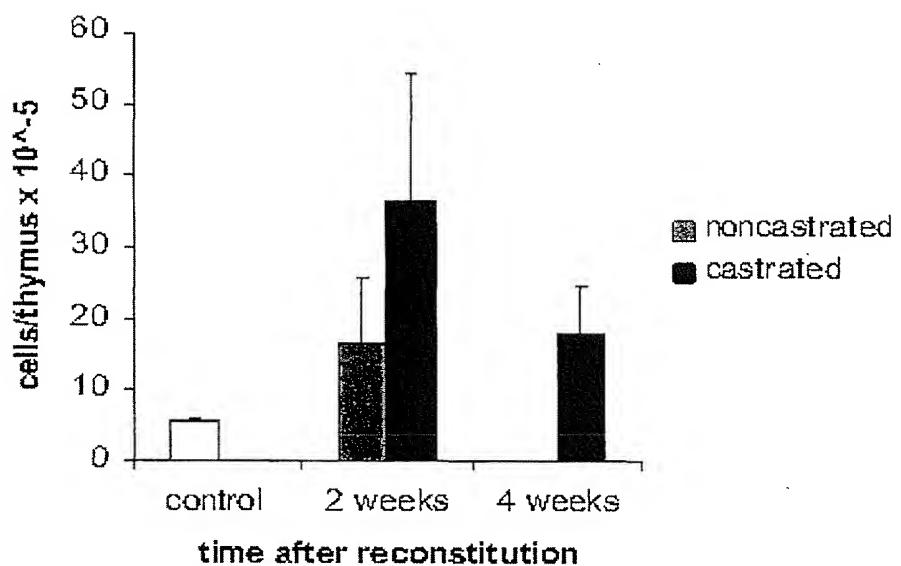
**Figure 16**

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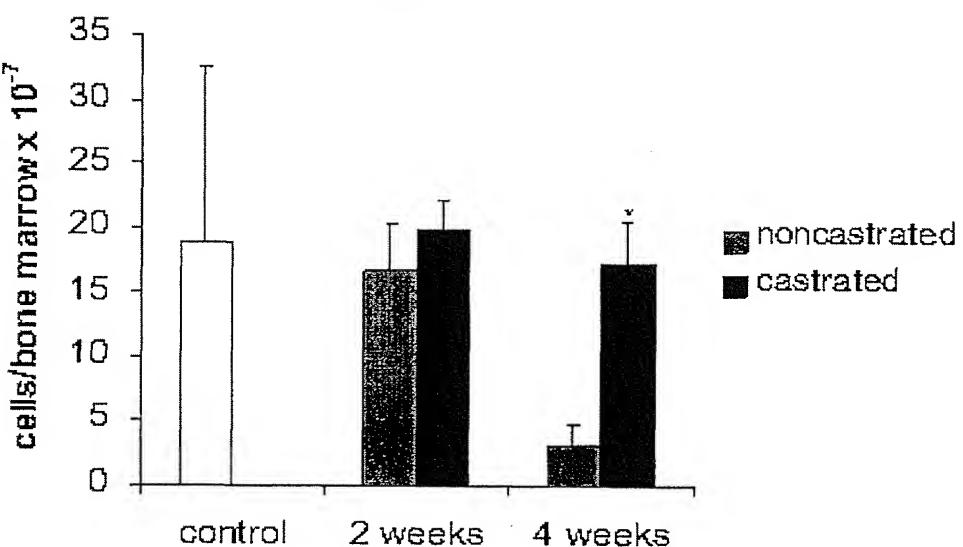
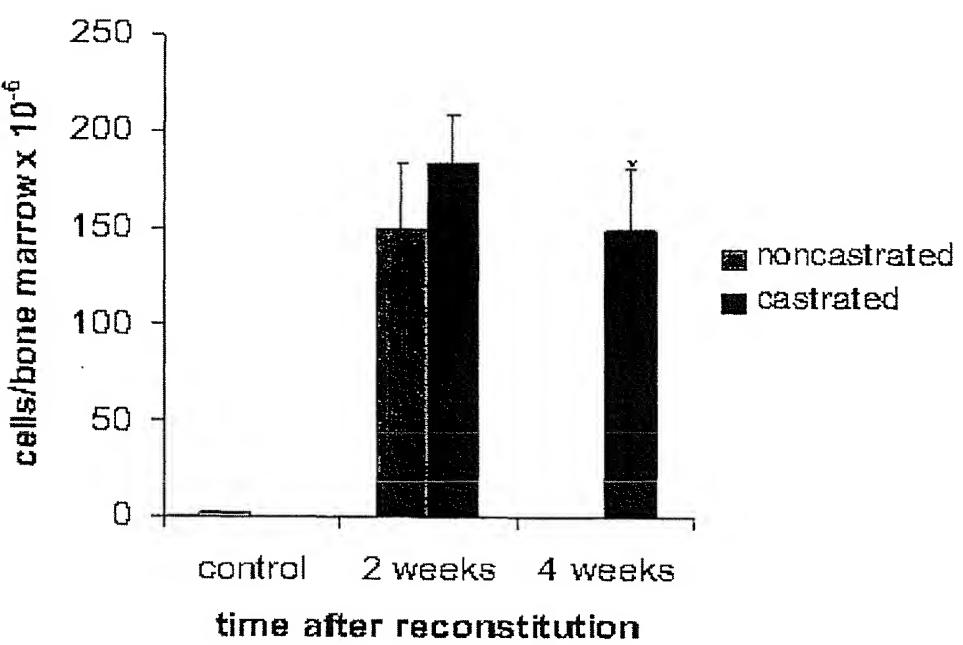


**FIGURE 17**

**FIGURE 18**

**A.****B.****FIGURE 19**

19/34

**A.****B.****FIGURE 20**

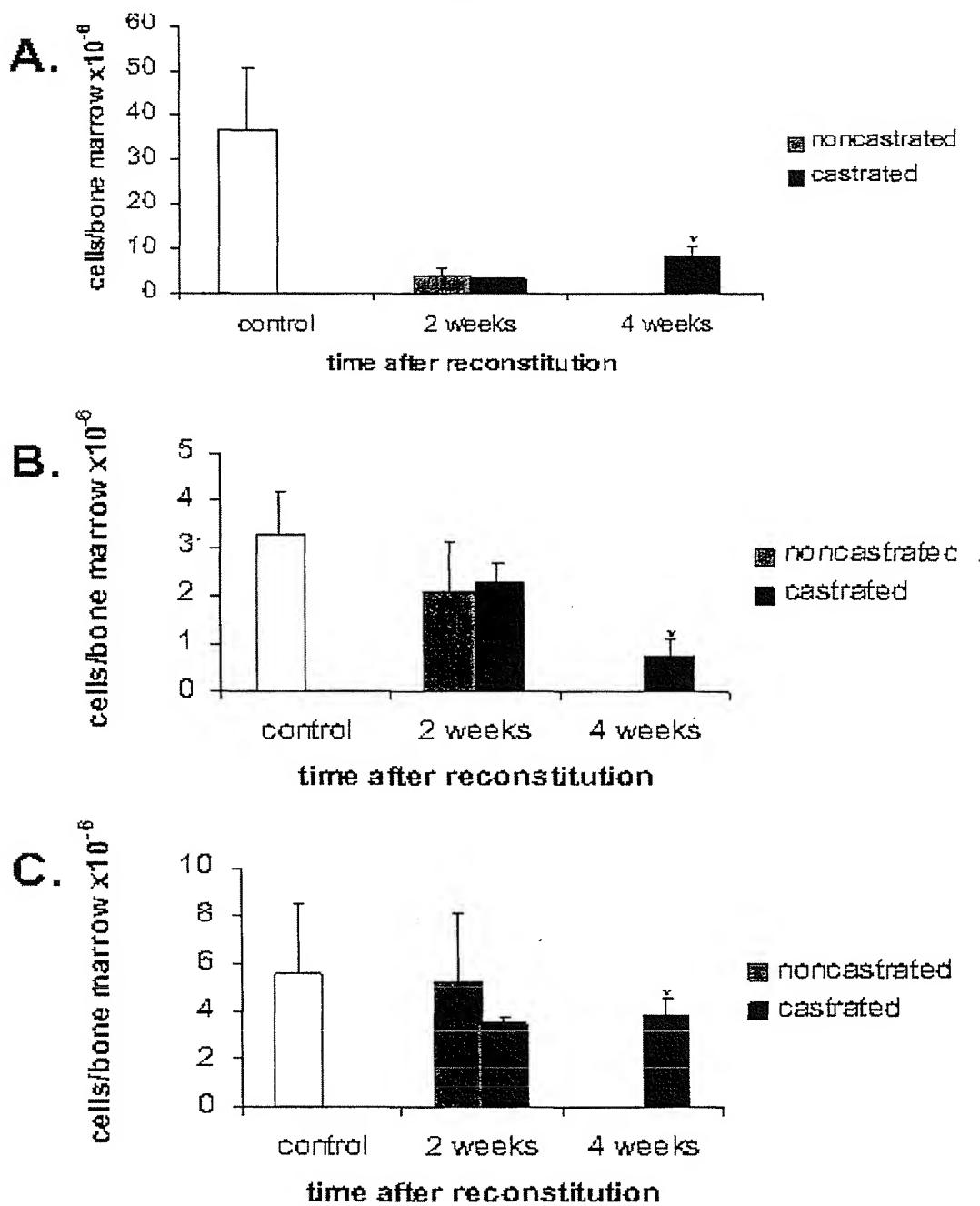


FIGURE 21

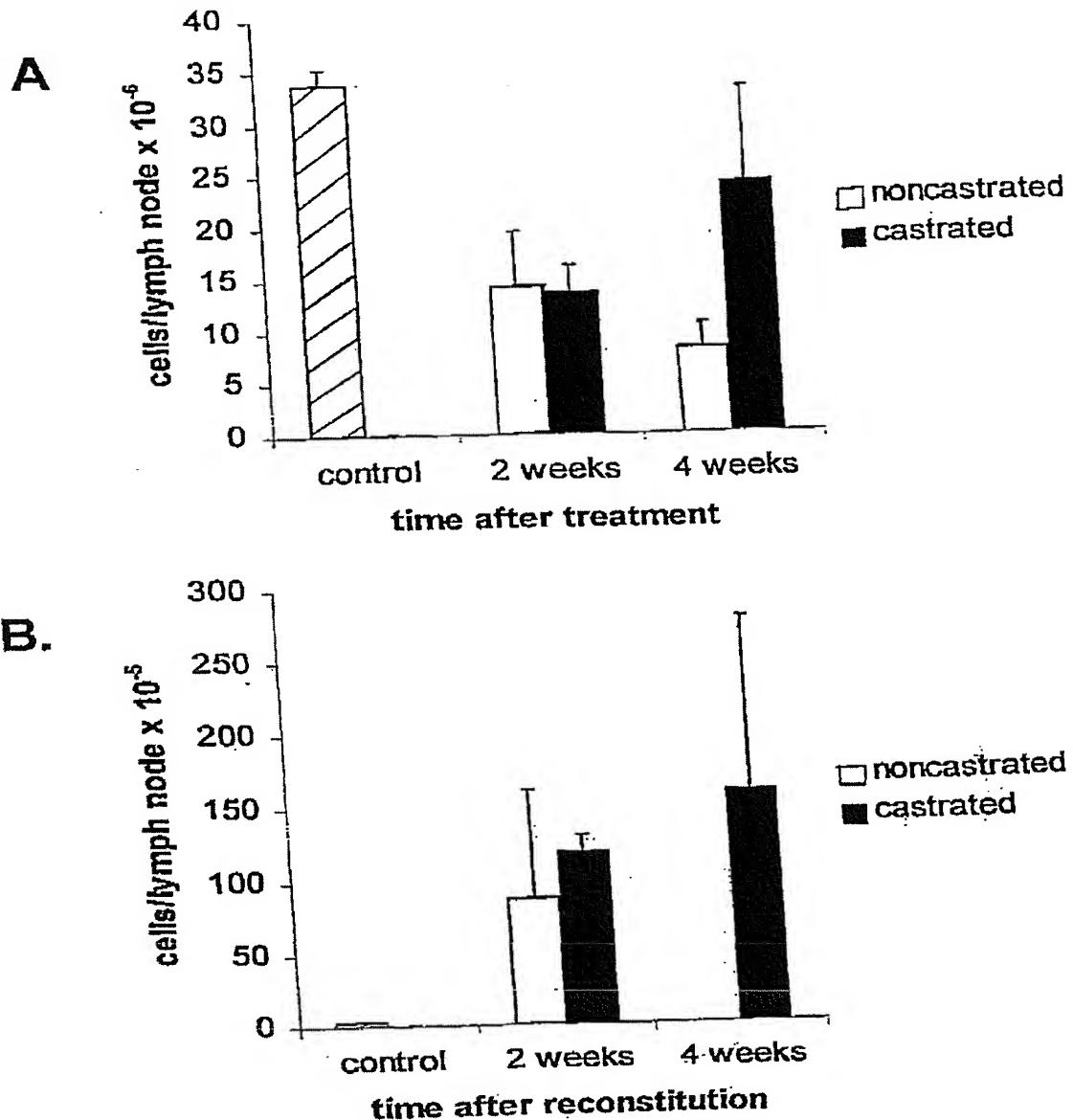
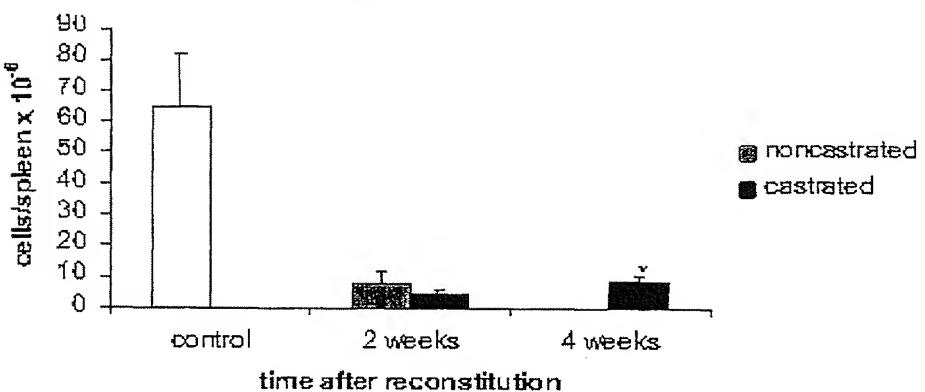
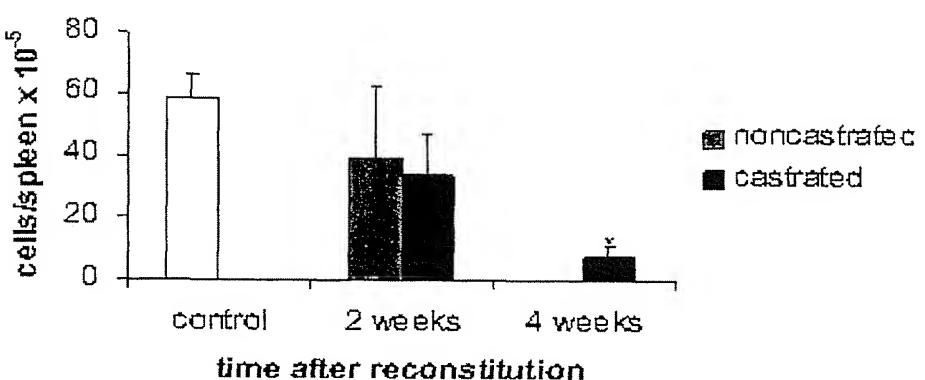
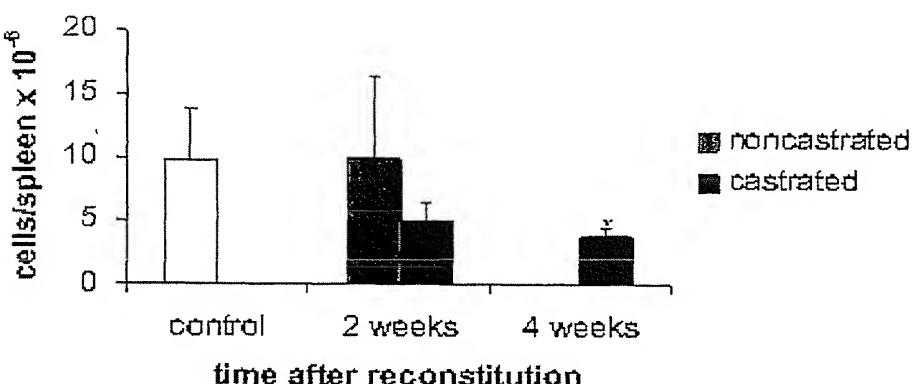
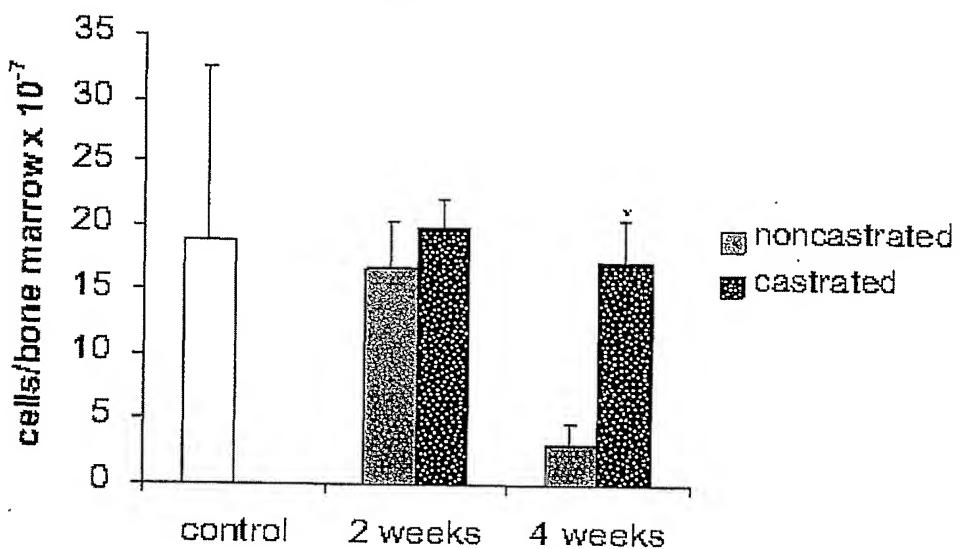
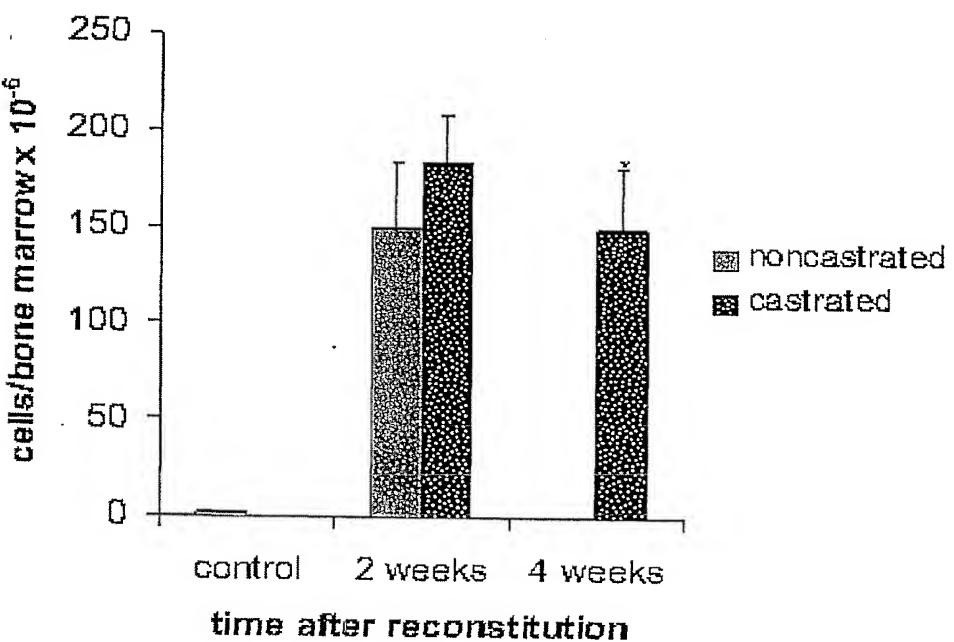
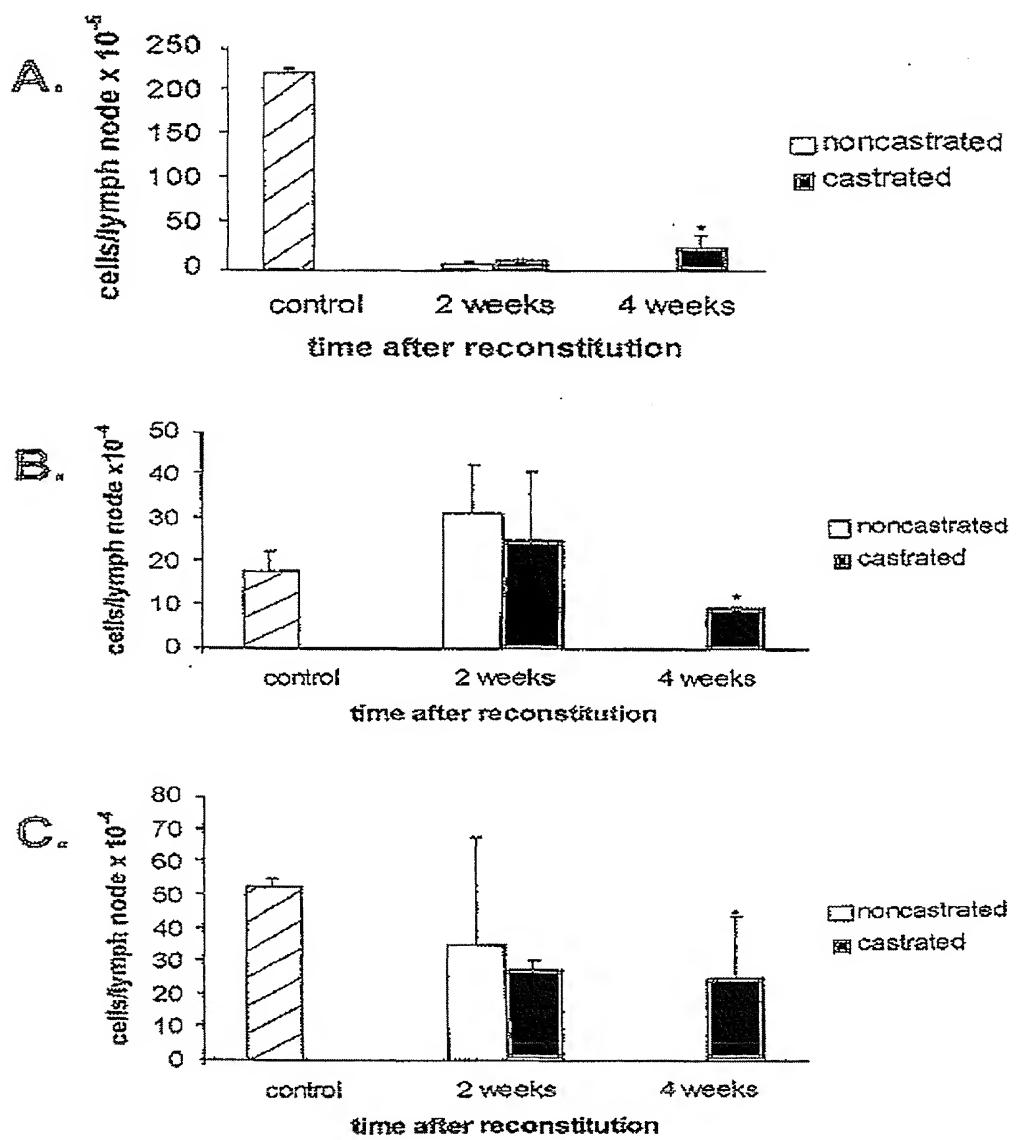


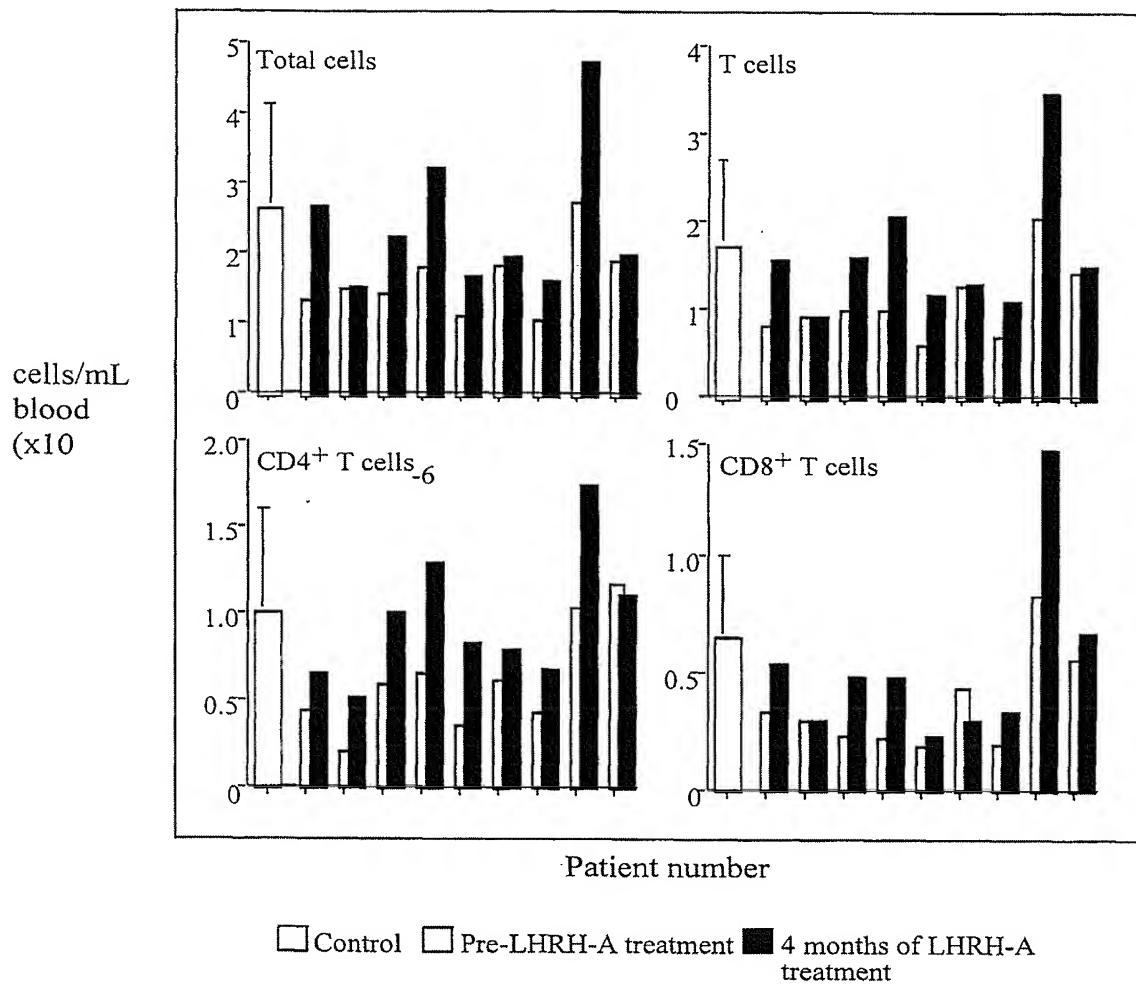
FIGURE 22

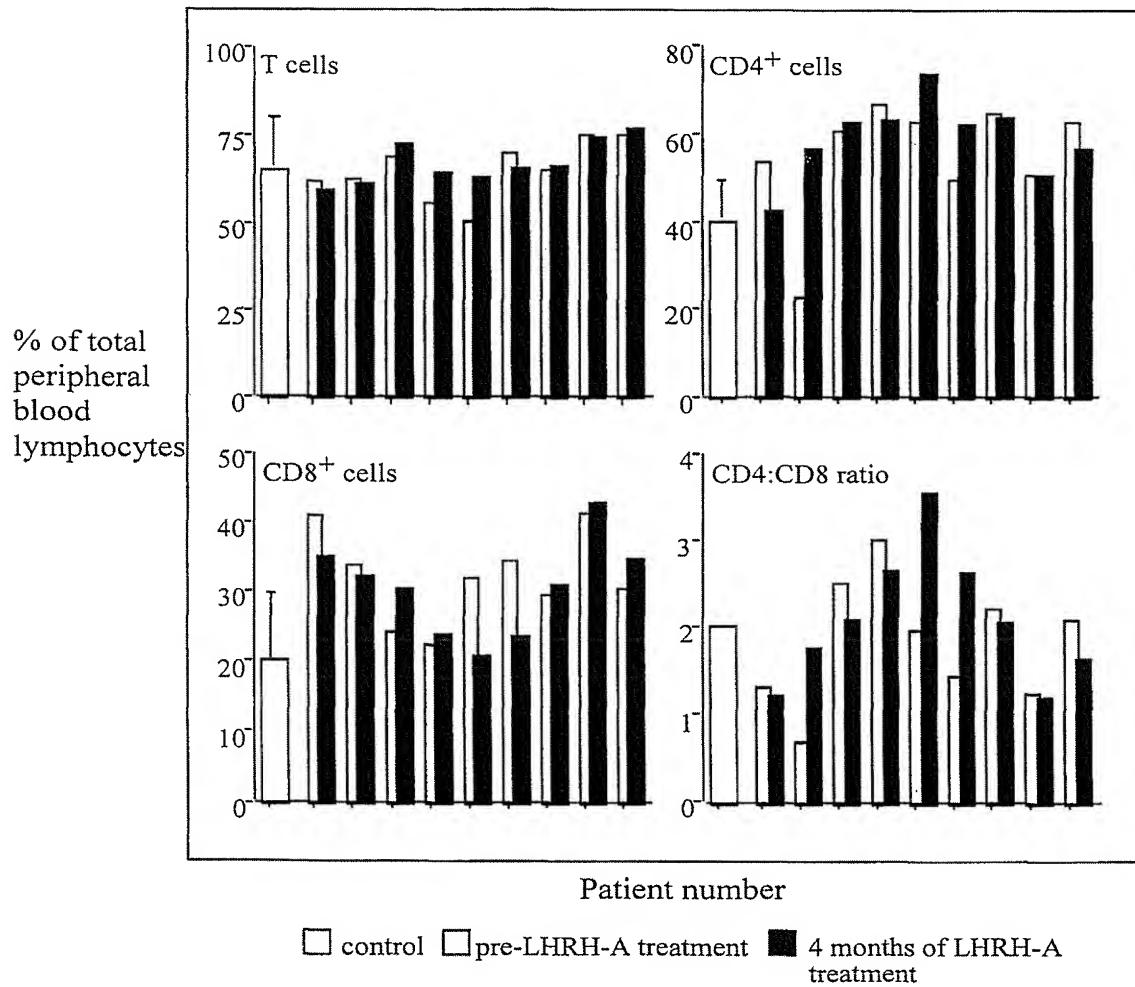
**A.****B.****C.****FIGURE 23**

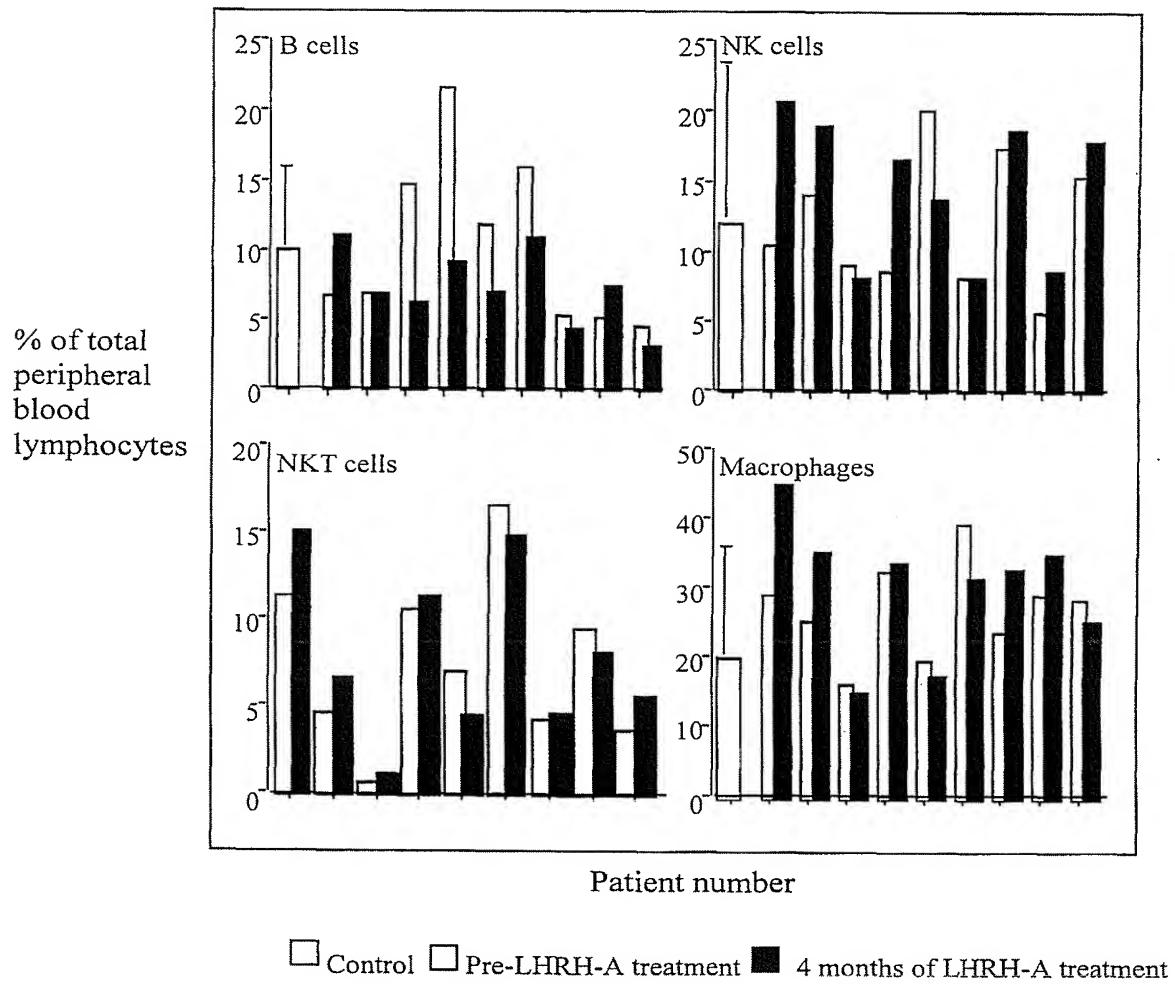
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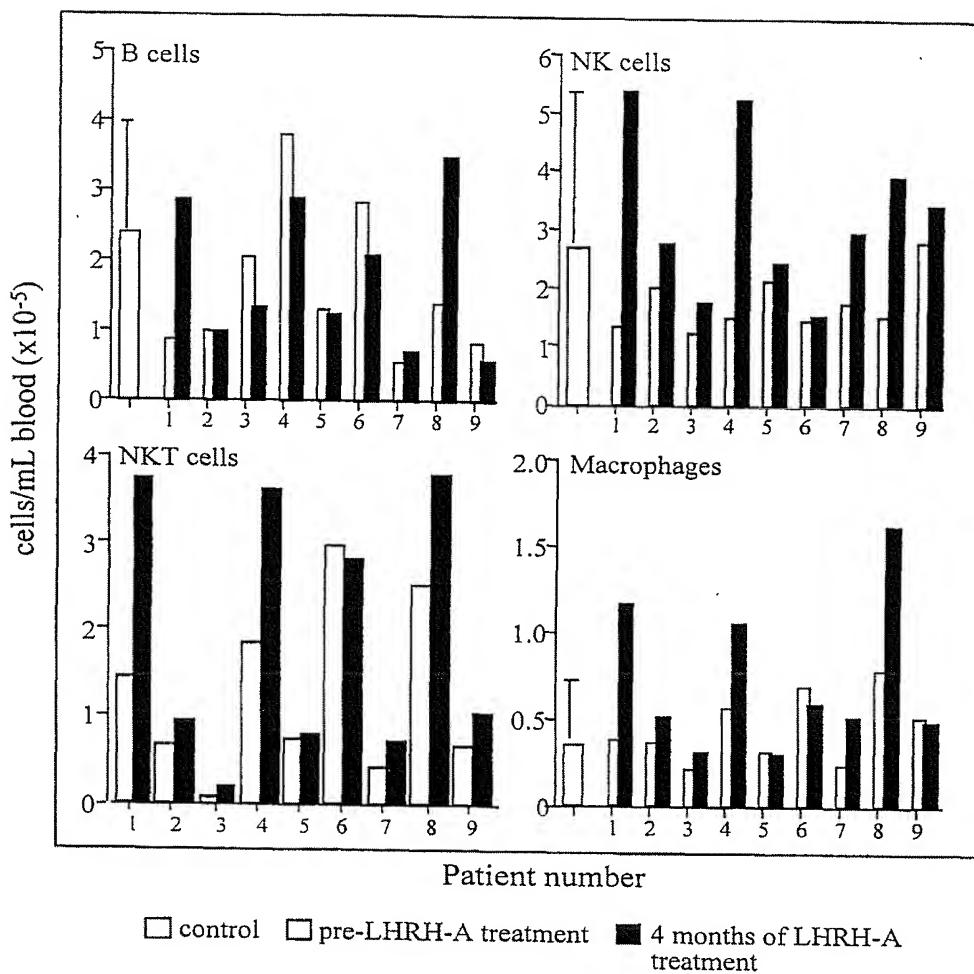
**A.****B.****FIGURE 24**

**FIGURE 25**

**FIGURE 26**

**FIGURE 27**

**FIGURE 28**

**FIGURE 29**

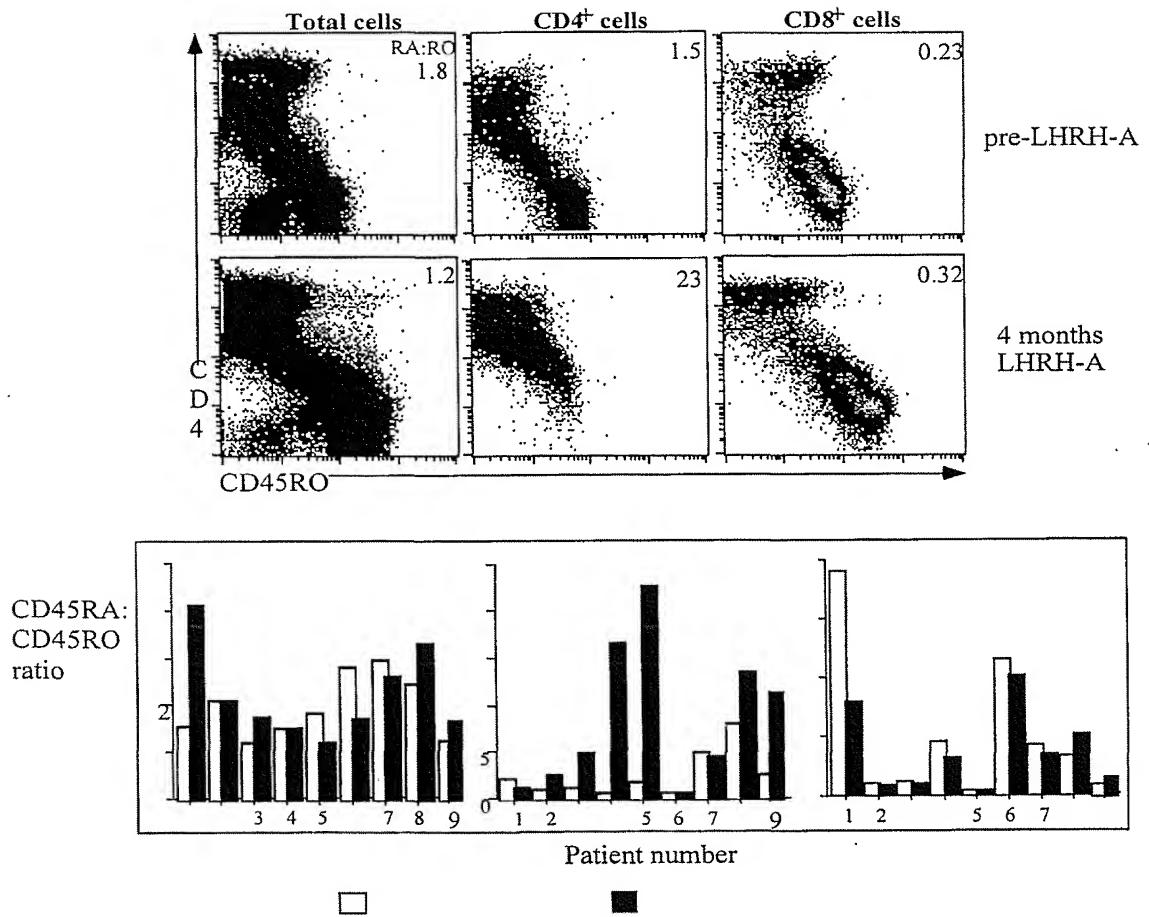
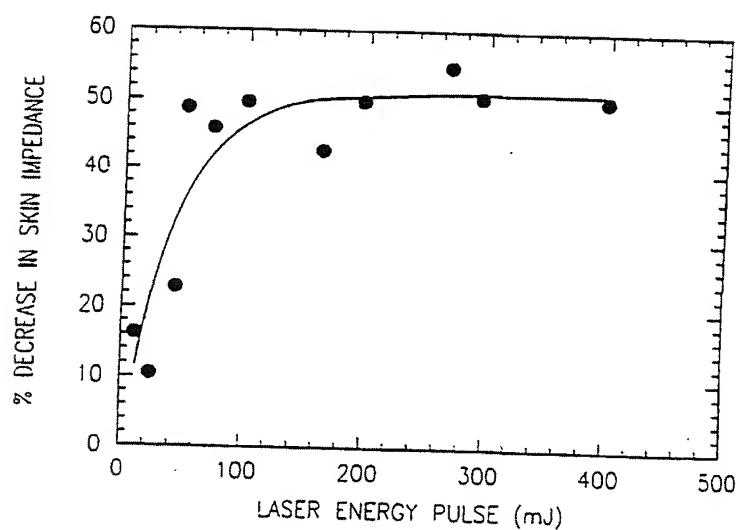
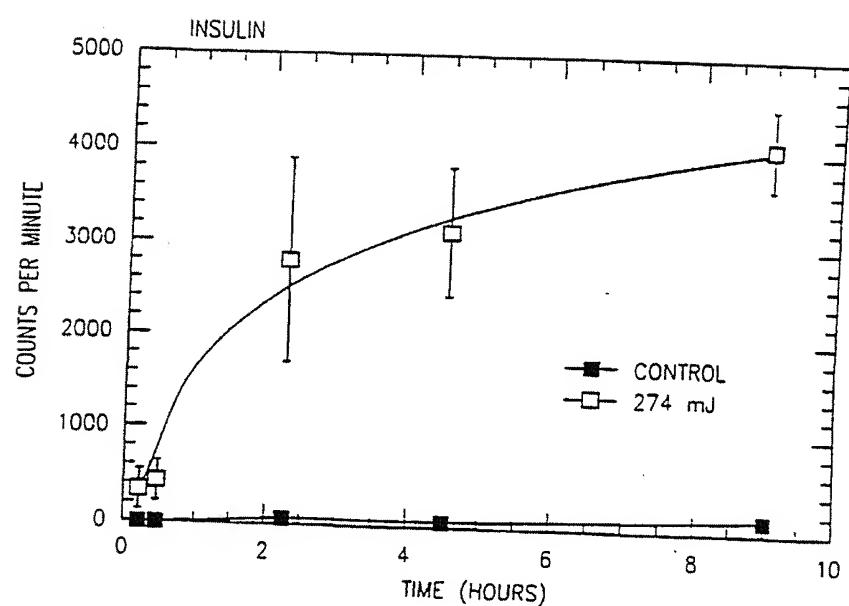


FIGURE 30

**FIGURE 31**

**FIGURE 32**

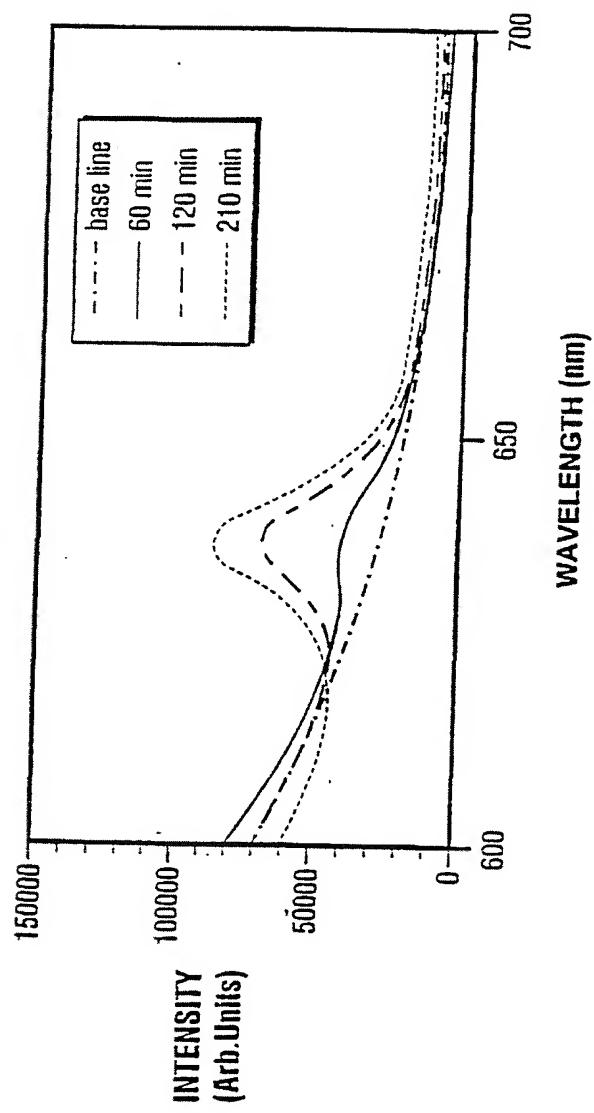


FIGURE 33

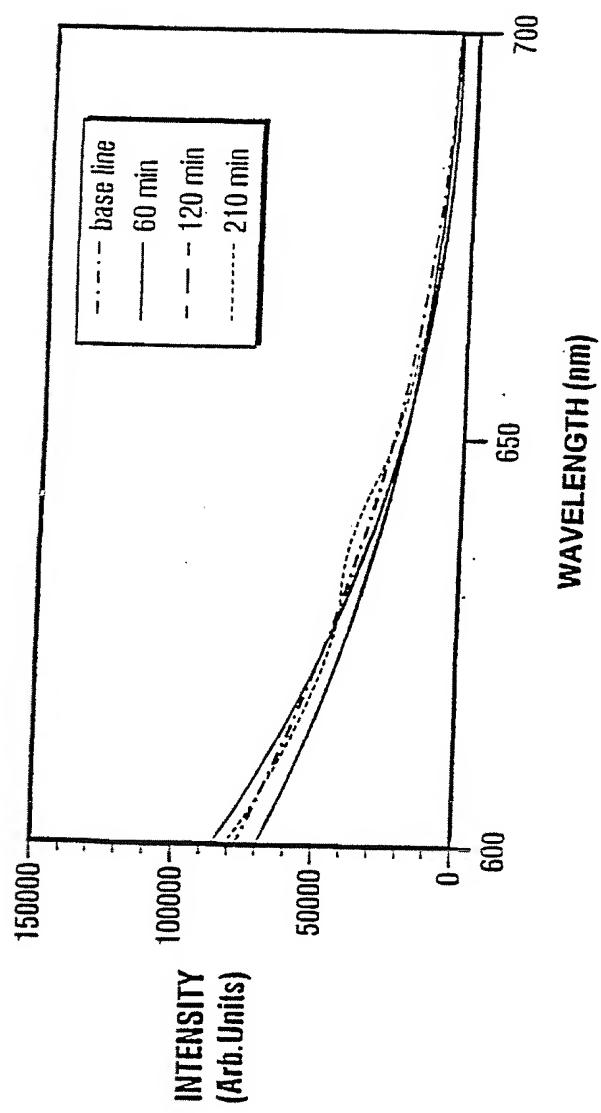


FIGURE 34

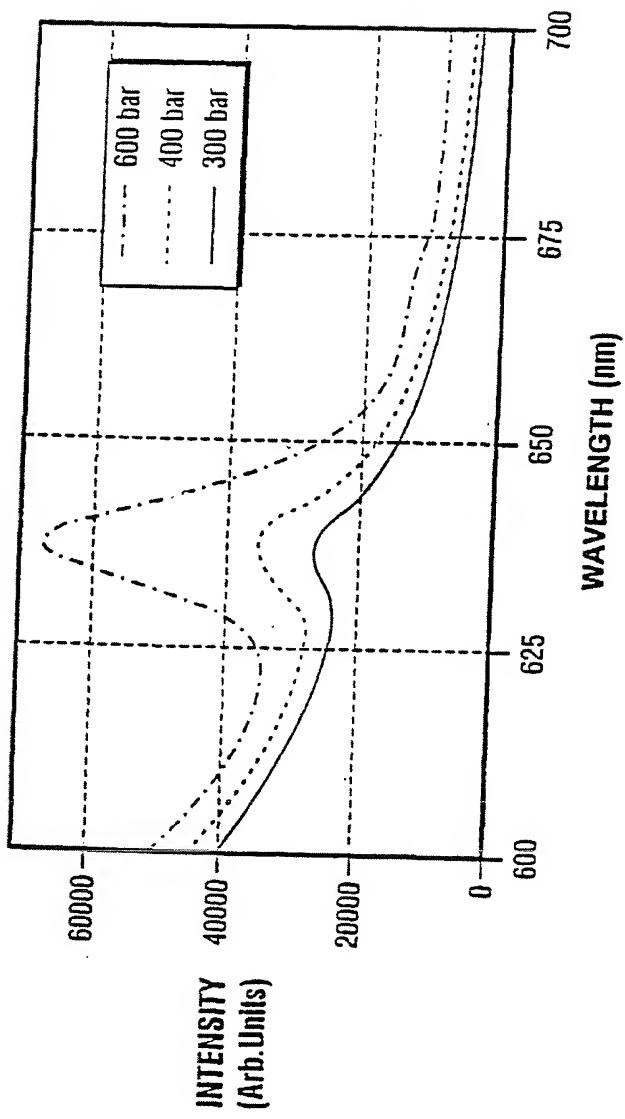


FIGURE 35